AD_____

Award Number: W81XWH-04-1-0124

TITLE: Centrosome-Based Mechanisms, Prognostics and Therapeutics in Prostate Cancer

PRINCIPAL INVESTIGATOR: Stephen J. Doxsey, Ph.D.

CONTRACTING ORGANIZATION: University of Massachusetts
Worcester, MA 01605-2377

REPORT DATE: December 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-12-2007	Final	1 DEC 2003 - 3O NOV 2007

4. TITLE AND SUBTITLE

Centrosome-Based Mechanisms, Prognostics and Therapeutics in Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0124

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Stephen J. Doxsey, Ph.D.

E-Mail: stephen.doxsey@umassmed.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Massachusetts
Worcester, MA 01605-2377

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Centrosomes are involved in mitotic spindle function and are abnormal in prostate tumors. This proposal investigated the role of centrosomes, centrosome proteins and other mitotic structures and processes in prostate cancer. Our results show that the centrosome protein pericentrin is present at the midbody, a structure involved in the final stage of cell division called cytokinesis, where it anchors PKA, PKB/Akt and PKC. Disruption of midbody anchoring (or depletion) of any of these kinases results in cytokinesis failure and aneuploidy, a hallmark of prostate carcinoma. A new asymmetric pathway involved in the completion of cytokinesis was identified and was characterized by asymmetric membrane trafficking, asymmetric positioning of the older centrosome and asymmetric inheritance of the midbody into one of the two daughter cells. Midbodies accumulated in subpopulations of cells in human prostate tumors and prostate cancer cell lines (PC3) but not in nontumor cells. Cancer cells that had midbodies were more aggressive in tumor assays. Midbody-containing cells were found in cells of stem cell niches and in cultured stem cells. We hypothesize that midbodies will serve as markers for prostate cancer 'stem cells' and possibly contri-bute to therapy-resistant prostate cancers.

15. SUBJECT TERMS
Centrosomes, Microtubules, Mitotic Spindle, Centrosome Instability, Aneuploidy, Checkpoint Control, Cytokinesis, Prognostics, Theraputics, PIN Lesions, Pericentrin, Centrolin, PKA, PKB, PKC, Cancer Progression, Gene Silencing

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	61	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

INTRODUCTION

Centrosomes are essential organelles that control many cellular functions (Doxsey et al, 2005a; 2005b). They are critical for organizing mitotic spindles and segregating chromosomes during mitosis. They also control cell shape and cell polarity, which are fundamental properties of epithelial gland organization. We and another group (Lingle et al, 1998) were the first to discover that centrosomes are structurally and numerically abnormal in nearly all malignant human prostate tumors (Pihan et al, 1998). This observation has important implications for cancer progression since it suggests that centrosome defects might contribute to cytologic anaplasia and genomic instability that so often accompany advanced prostate cancer. Support for this idea came from our recent observations that centrosome defects, cytologic anaplasia and genomic instability could be artificially induced in nontumor cells by elevating the levels of a single centrosome protein called pericentrin and that pericentrin was elevated in many prostate carcinomas and pre-invasive lesions (Pihan et al, 2001; Pihan et al, 2003). Based on these observations, and the knowledge that clinically aggressive prostate carcinoma (high Gleason grade) exhibits significant anaplasia, epithelial de-differentiation and genomic instability, we proposed an innovative hypothesis: that centrosome dysfunction may be a critical factor in prostate cancer progression. We believe that progressive centrosome dysfunction is the first biologic factor identified that can fully explain most of the phenotypic changes characteristic of prostate carcinomas during their progression from clinically indolent forms (majority) to clinically aggressive forms (minority). The specific aims of the original proposal were designed to test several features of this model. 1. Are centrosome defects present in early prostate cancer and can they predict aggressive disease? 2. Do pericentrin's oncogenic features result from the interaction with protein kinases? 3. Can prostate tumor cells be arrested in the cell cycle by overexpression of a domain of pericentrin that drives cells out of cycle? We anticipate that this work will lead to new and powerful prognostic markers as well as novel cancer-specific therapeutic targets for clinically aggressive prostate cancer, the form of prostate carcinoma that is clinically most critical in terms of diagnosis, treatment and health care expenditure.

REPORT BODY

In this final report, we summarize progress made on this proposal. During the course of these studies, we made some unexpected discoveries and in some cases these new directions were pursued. As a result, some aims were modified to accommodate these changes. In other cases, results other than those predicted were obtained and pursuit of the stated goals were curtailed. Details of these changes are described below.

Aim 1. Progress on Aim 1 has not yet been possible due to the dissolution of our collaboration with Walter Reed Hospital, which was precipitated after the 9/11 disaster. We also had hoped to obtain relatively rare PIN samples with follow up from Dr. Albrecth Reith at The Norwegian Radiumhospital, Institute for Cancer Research, Norway. However, a fraud scandal resulted in a shut down of all outside collaborations (see appendix 'radiumhospital'). We hope to resume work on this aim in the future. We feel that progress made in new directions compensate for the lack of progress on Aim 1 (see below).

Aim 2. We originally showed that elevation of the levels of the centrosome protein pericentrin induced centrosome defects, genetic instability and anaplasia in human prostate cells (Pihan et a, 1998). We now show that the mechanism by which pericentrin accomplishes this is complex. It is in part through misregulation of PKC as we originally showed (Chen et al, 2004) with contributions from PKA and PKB/Akt. We have completed the construction of mutants that lack pericentrin binding (Task 2b) and they will be used in future studies. We have examined growth of pericentrin-expressing cells in mouse prostate glands (Task 2a) and we did not find a significant increase in tumor growth. Tumor volumes (1/2(length [mm]) x (width [mm])^2 were 72.4+/-6.1 mm^3 for control cells versus 64.3+/-3.3 mm^3 for pericentrin-expressing (n=15 mice/group). In fact, the results suggested that tumor growth was retarded slightly in pericentrin-expressing cell injections, a result

inconsistent with our original in vitro assays showing growth in soft agar was increased (Pihan et al, 2001). Until we get a better understanding of this result, we have postponed analysis of the pericentrin mutants in vivo (Task 2c, d). One possible reason for this unexpected result was that pericentrin interacts with many other proteins. Through protein interaction screens we identified several other pericentrin-interacting proteins that contribute to the aneuploidy phenotype (e.g. gamma tubulin, NuRD, centriolin, MT1-MMP, IFT proteins, polycystin2). We expect that the mechanism of pericentrin-induced aneuploidy will be very complex and will involve many molecules other than PKA/B/C. We hope to investigate the contribution of each pericentrin-interacting protein that binds to aneuploidy and prostate tumorigenesis.

During the course of these studies, we made the novel discovery that PKA, PKB and PKC were localized to midbodies and anchored there by pericentrin (Chen et al, 2004, Fig. 1, 2, 5, see below in 'Supporting Data' section before Appendix). Moreover, pericentrin depletion by RNA interference mislocalized the kinases from this site (Fig. 5, data not shown). In addition, we showed that disruption of the kinases themselves by RNA interference or dominant-negative expression, caused cytokinesis failure and that disruption of pericentrin's interaction with PKA and PKC give the same phenotype (Fig. 3, 4--two pages). In all cases, cells fail to divide after replicating both DNA and centrosomes creating polyploid with amplified centrosomes. The extra centrosomes can, in turn, form multipolar spindles that missegregate chromosomes and lead to profound genetic instability. This demonstrates a previously unappreciated role for all three kinases in cytokinesis and aneuploidy. This work is currently being submitted for publication.

Aim 3. We previously showed that depletion of pericentrin and another of its binding partners, centriolin, induced G1 arrest and that this cell cycle arrest could be induced by overexpression of domains of pericentrin and centriolin (Mikule et al, 2007, data not shown). To determine if this cell cycle arrest function could be used to block prostate cancer cell division and serve as an anti-prostate tumor strategy, we constructed centriolin and pericentrin plasmids that blocked the cell cycle in normal cells (Task 3a, b). However, we unexpectedly found that expression of these constructs in prostate cancer cells that lacked functional p53 (PC3) did NOT induce arrest as expected (Task 3b). Moreover, prostate cancer cells with wild type p53 also did NOT arrest (e.g. LnCAP). We have subsequently discovered that the inability of prostate cancer cells to undergo cell cycle arrest is due to abrogation of p38, p53, p21, cyclin A, Cdk2 or other unidentified genes in this new pathway (Mikule et al, 2007). Although cell cycle arrest by centriolin expression may not be a method to stop prostate cancer cells from cycling (dividing) and thus block tumor growth, we did notice that the cells that continue to cycle in the presence of centriolin or pericentrin domains died at a higher rate than controls (14.5% vs. 3.4% in n=4 experiments) but this was not significant over time. Because prostate cancer cells did not arrest as expected, progression to the final step of Task 3 (part c) was not initiated.

However, during the course of the studies on centriolin overexpression and depletion (Task 3), we identified a role for the protein in a novel aspect of cytokinesis (vesicle mediated secretion) and showed that disruption of endogenous centriolin led to cytokinesis failure and aneuploidy (Gromley et al, 2005, appendix), a hallmark of prostate carcinoma. Perhaps more interesting was that this process required multiple asymmetric events (Fig. 6, see below in 'Supporting Data' section). Even more interesting was the observation that the midbody, a singular structure made during the process of cell division and required for cell division, was not lost as previous studies had suggested but was retained by *one* of the two daughter cells (see Fig. 6). Thus, the two cells 'born' after cell division are not equal—one inherits a large structure comprised of hundreds of proteins, the midbody. Moreover, this cell inherits midbodies after each successive division and accumulates them (see Fig. 6)! When we examined human prostate tumors in vivo and human prostate cancer cell lines in vitro, we found midbodies in a subpopulation of cells (Fig. 7). Midbodies were in the cytoplasm of cells and they numbered up to 7 in some PC3 cells. We reasoned that the subset of prostate cancer cells might have special properties; perhaps they were 'stem-like' or 'cancer initiating cells'. To test this, we examined human and mouse stem cells in organs and stem cell lines in vitro and found that midbodies were present in normal stem cells of the testes, gut, hair

follicle and other organs (Fig. 8-11, Table 1). They were also present in many other cancer cell lines but NOT in matched non-cancer cell lines or in normal non-tumor, non-stem cells in vivo. We constructed a GFP-MKLP1 HeLa cell line and we are making a GFP-MKLP1 PC3 cell line. We have flow sorted the GFP-MKLP1 HeLa line and obtained a population with a high percentage of midbody-containing cells. These cells divide more rapidly and make more colonies in agar than the midbody low population (8-10-fold increase). A proposal containing aspects of this work (which were initiated during the course of this proposal) was submitted to the PRCP of the D.O.D. and received a score of Excellent but was not funded (PC073330 and reviews in appendix).

SUPPORTING DATA

(see the following pages)

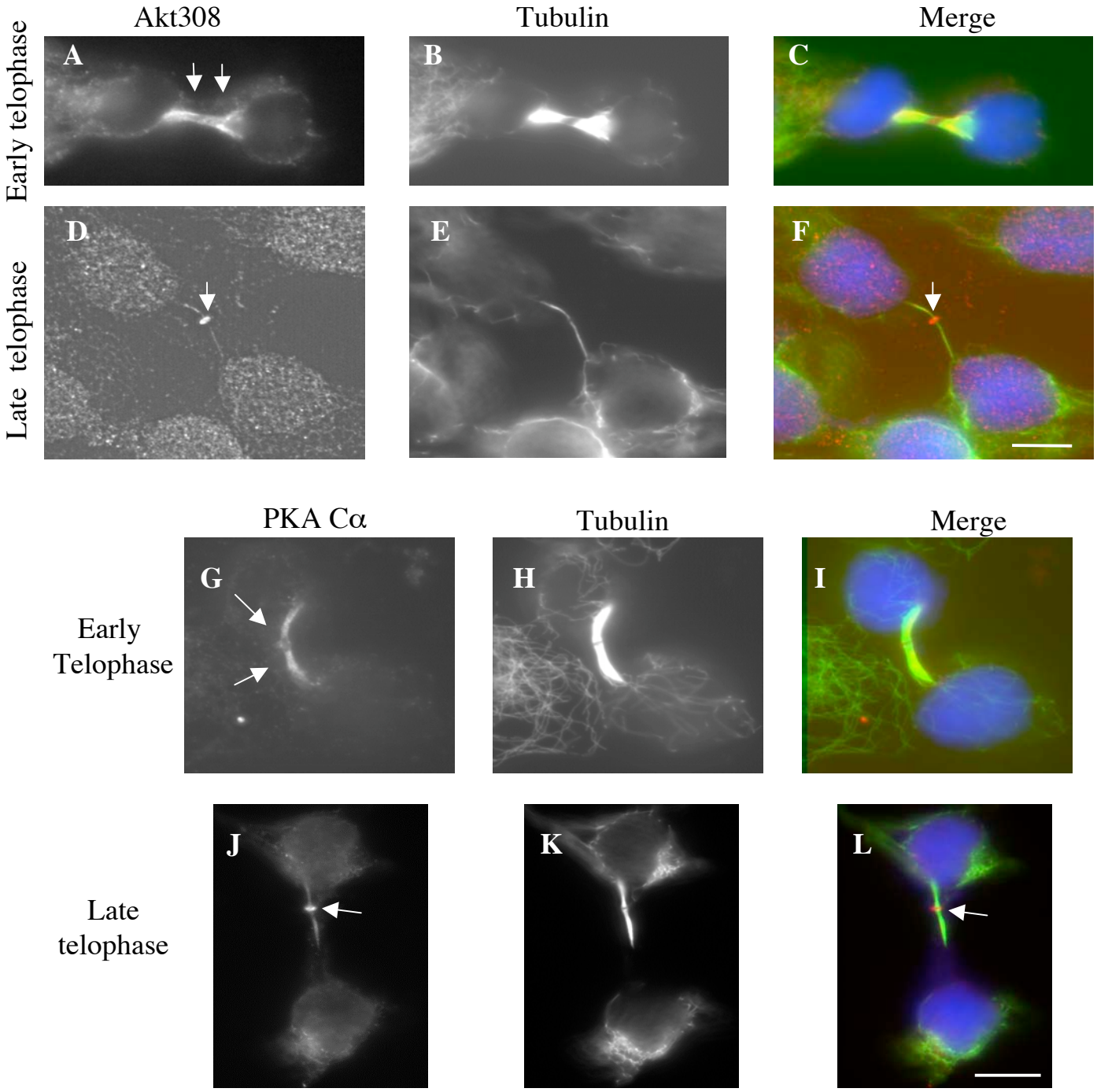


Fig 1. PKA Cα and PKB localize to midbody: Micrograph shows the staining of HEK 293 cells with anti Akt308 (PKB) (red, A, D), anti PKA C α (red, G, J), tubulin (green B, E, H, K) and DAPI (blue) for DNA (merge: C, F, I, L). Bar in F, 10 μm for A-F and Bar in L, 10 μm for G-L.

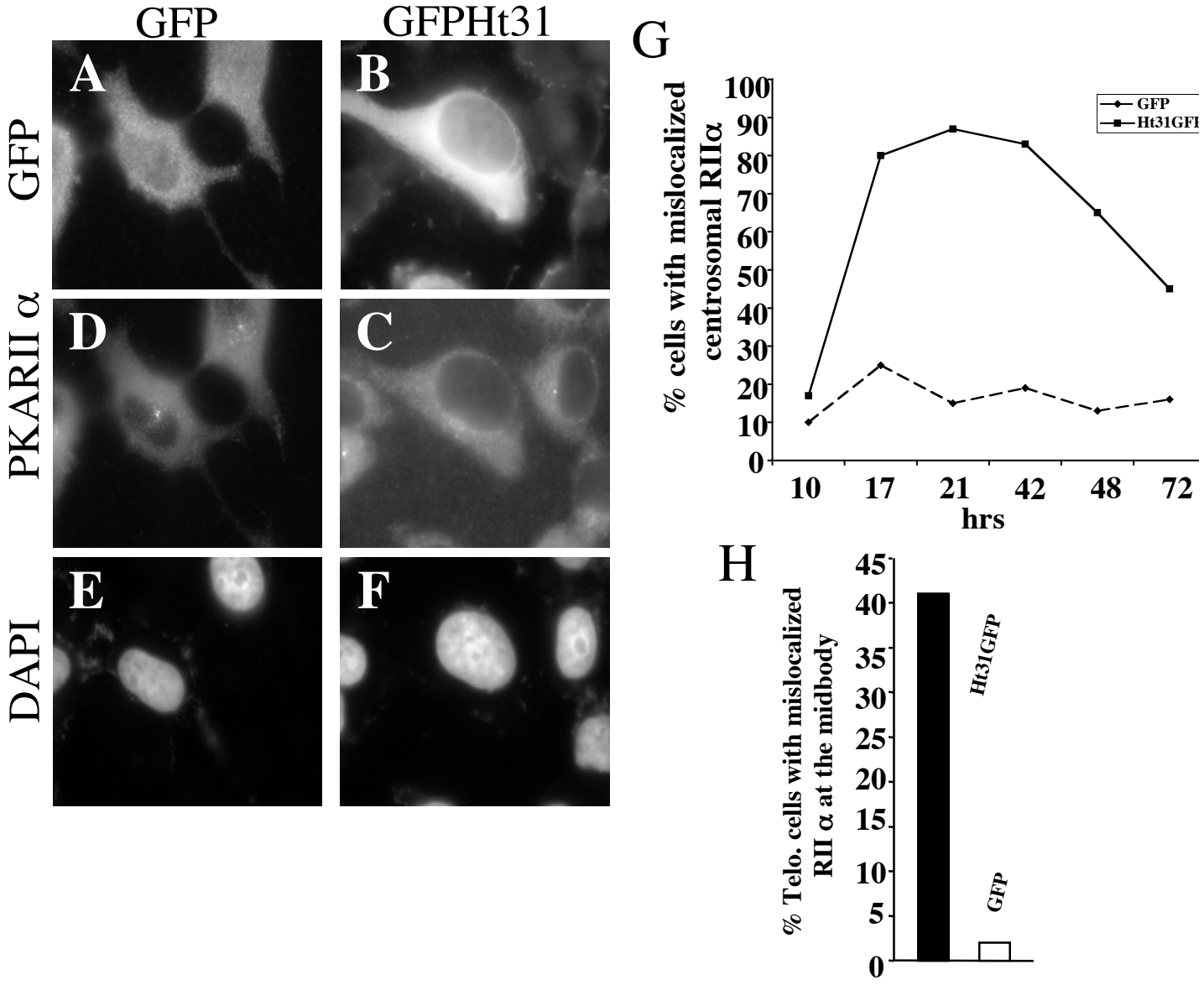


Fig 2. Mislocalization of PKA-RII α (PKA regulatory domain) at the centrosome and midbody in Ht31GFP (pericentrin anchoring domain) overexpressing cells: Images shows the GFP (A) and Ht31GFP (B) expressing HEK 293 cells staining of anti PKA RII α (red: D, C) and DAPI (E, F). Bar in F, 10 μm for A-F. ***Note loss of PKA signal in C.*** Quantification of the results are shown in (G): A time course analysis of the RII α mislocalization at the centrosome, and in (H): mislocalization of RII α at the midbody. The experiments were repeated twice at two time points and about 150 cells were counted at each time point in this time course analysis (G).

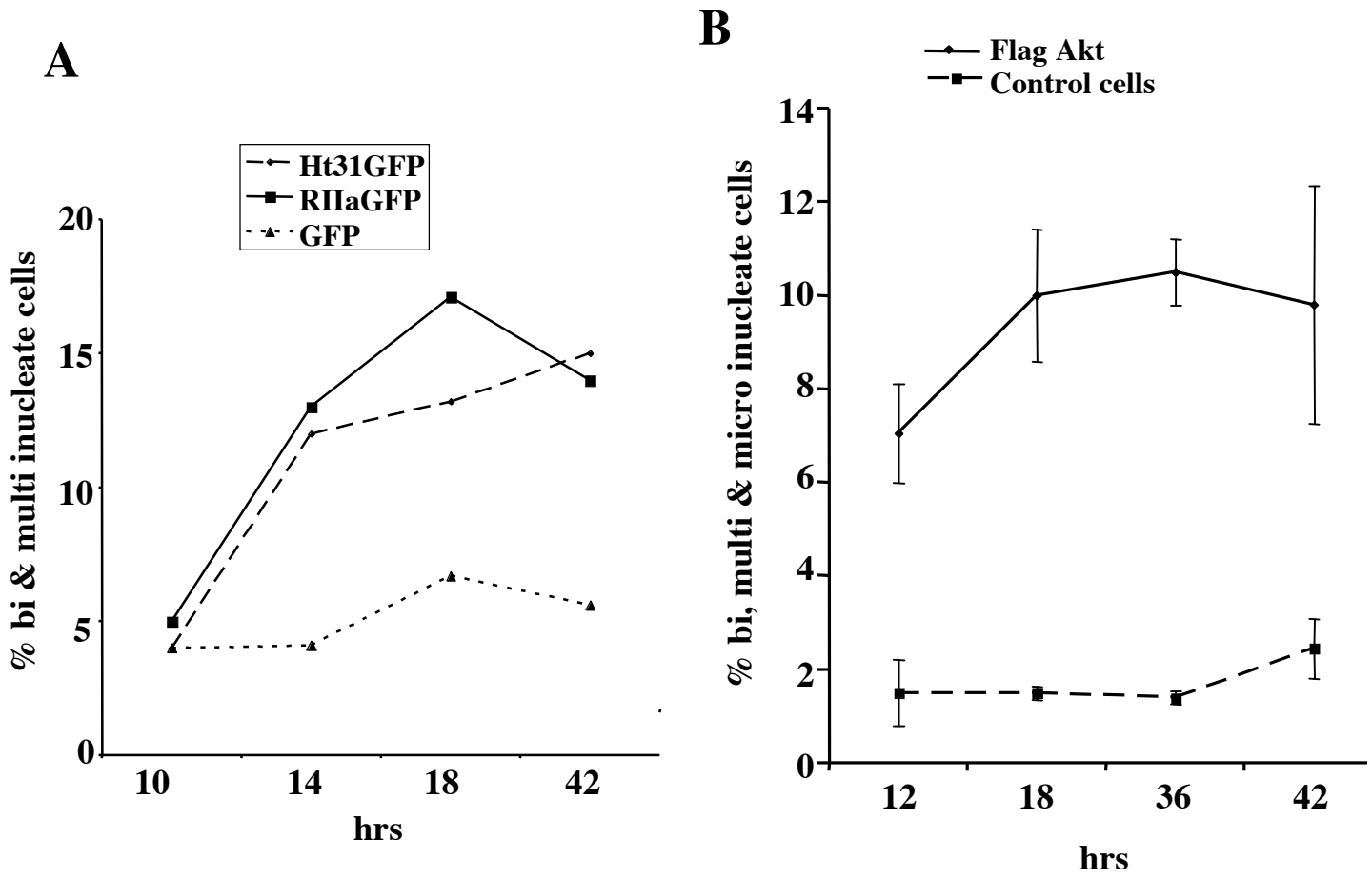
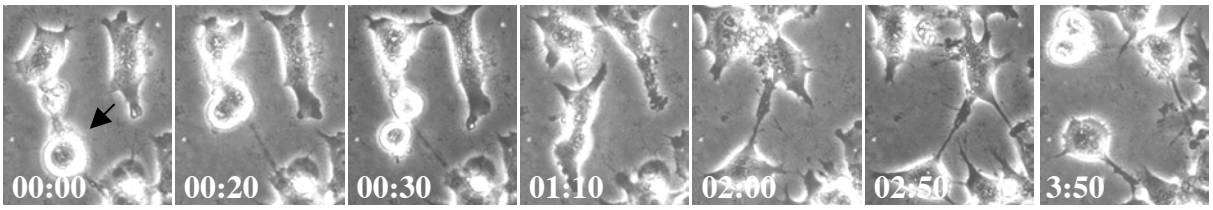
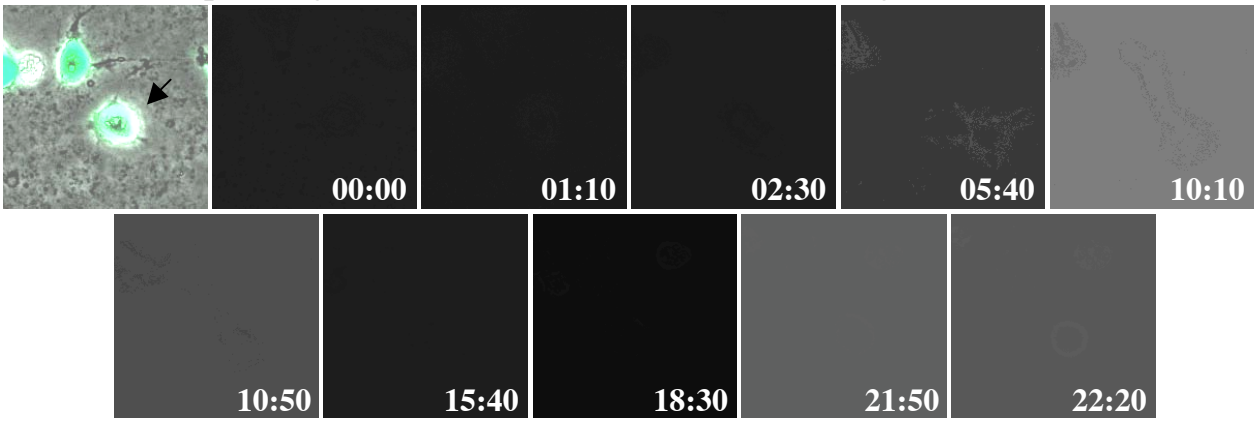


Fig 3. Overexpression of Ht31GFP (pericentrin anchoring domain) and Flag-Akt mislocalizes PKA C α and Akt from the midbodies, respectively, and induces bi and multi nucleated cells. Quantitation of the results are shown in (A): Ht31GFP overexpressing HEK 293 cells and (B): Flag-Akt overexpressing COS cells. The experiments were repeated thrice at two time points for both the constructs and in this time course analysis 200-400 cells were counted at each time point.

A. GFP-expressing cell (control) divides after 20 h (normal timing - 4 h)



B. GFP-Ht31-expressing cell divides after 20 h (normal timing: 2-4 h)



C. GFP-Ht31-expressing cell fails division after 7 h (forms binucleate)

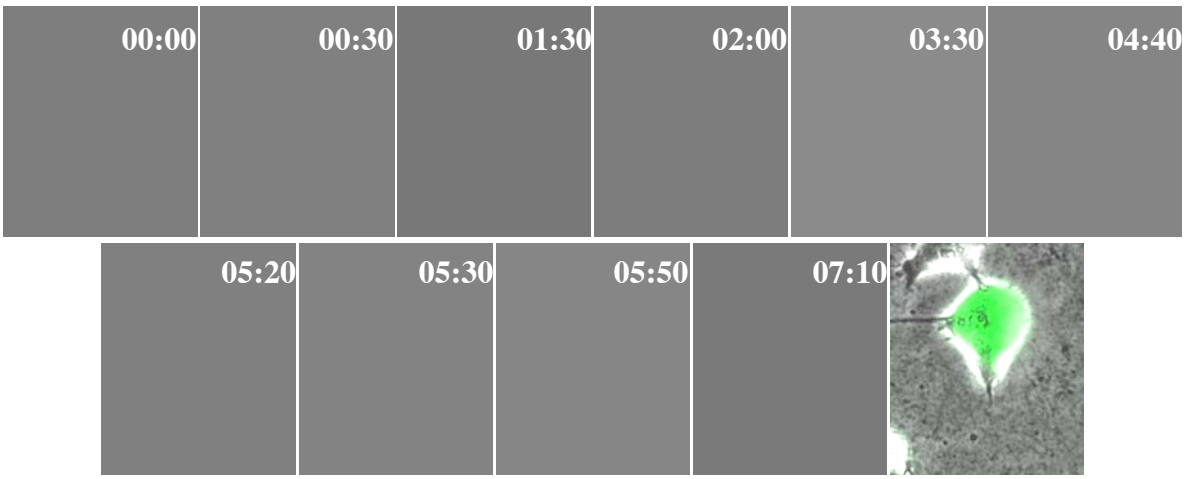


Fig 4. Time-lapse images of Ht31GFP overexpressing HEK 293 cells show defective cytokinesis. (A) A GFP expressing cell goes through nuclear envelope breaking, metaphase, anaphase, forms midbody and completes the final cytokinesis event in normal timing (4-6 h after metaphase). (B) Ht31GFP expressing cells remained attached for prolonged periods of time through persistent intercellular connection for 16 h on average. The cell cleaves, one of the daughter cell round up for next division. (C) Ht31GFP expressing cell goes through normal metaphase, anaphase and telophase but did not divide into two daughter cells, instead became binucleate cell.

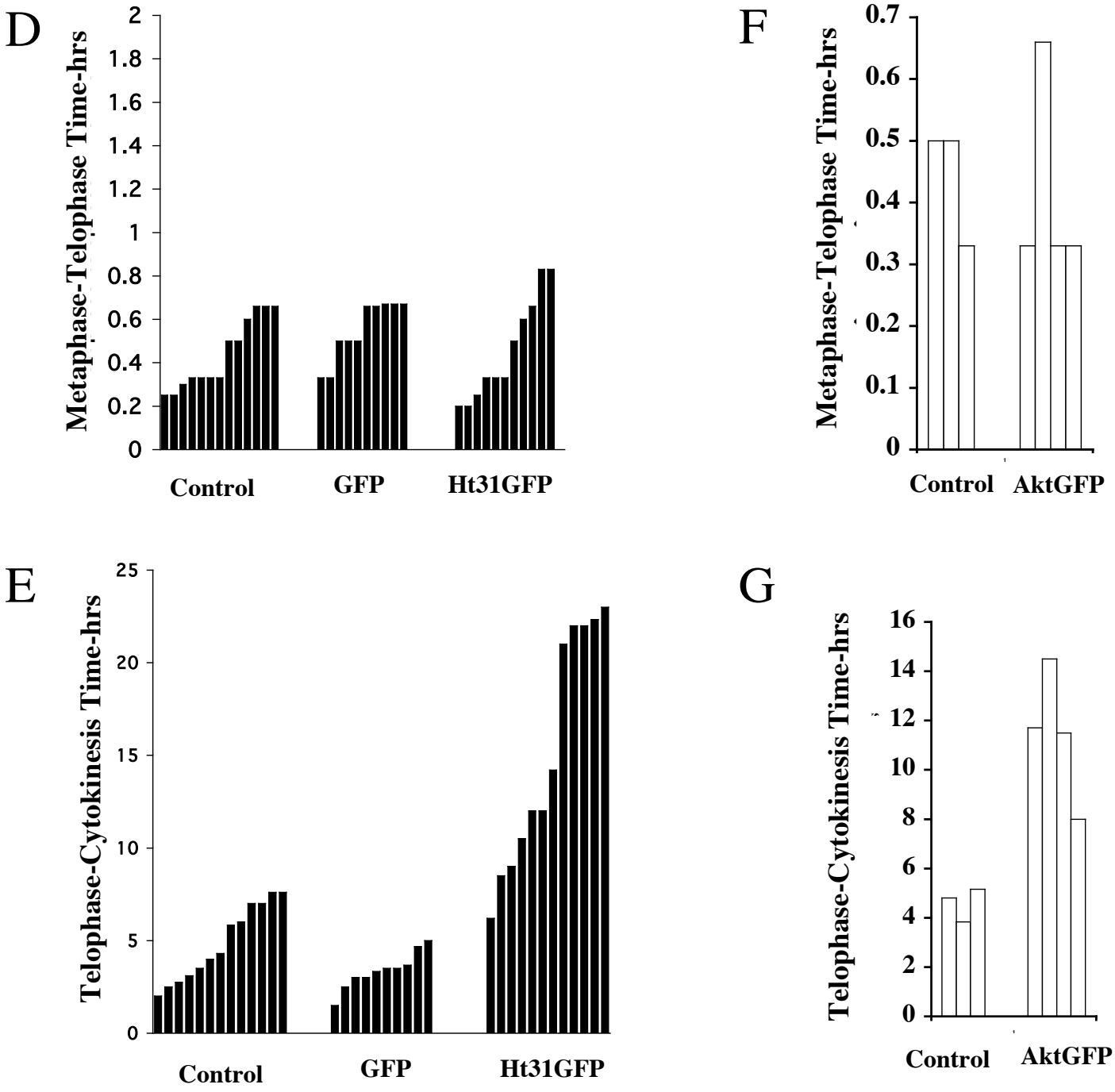
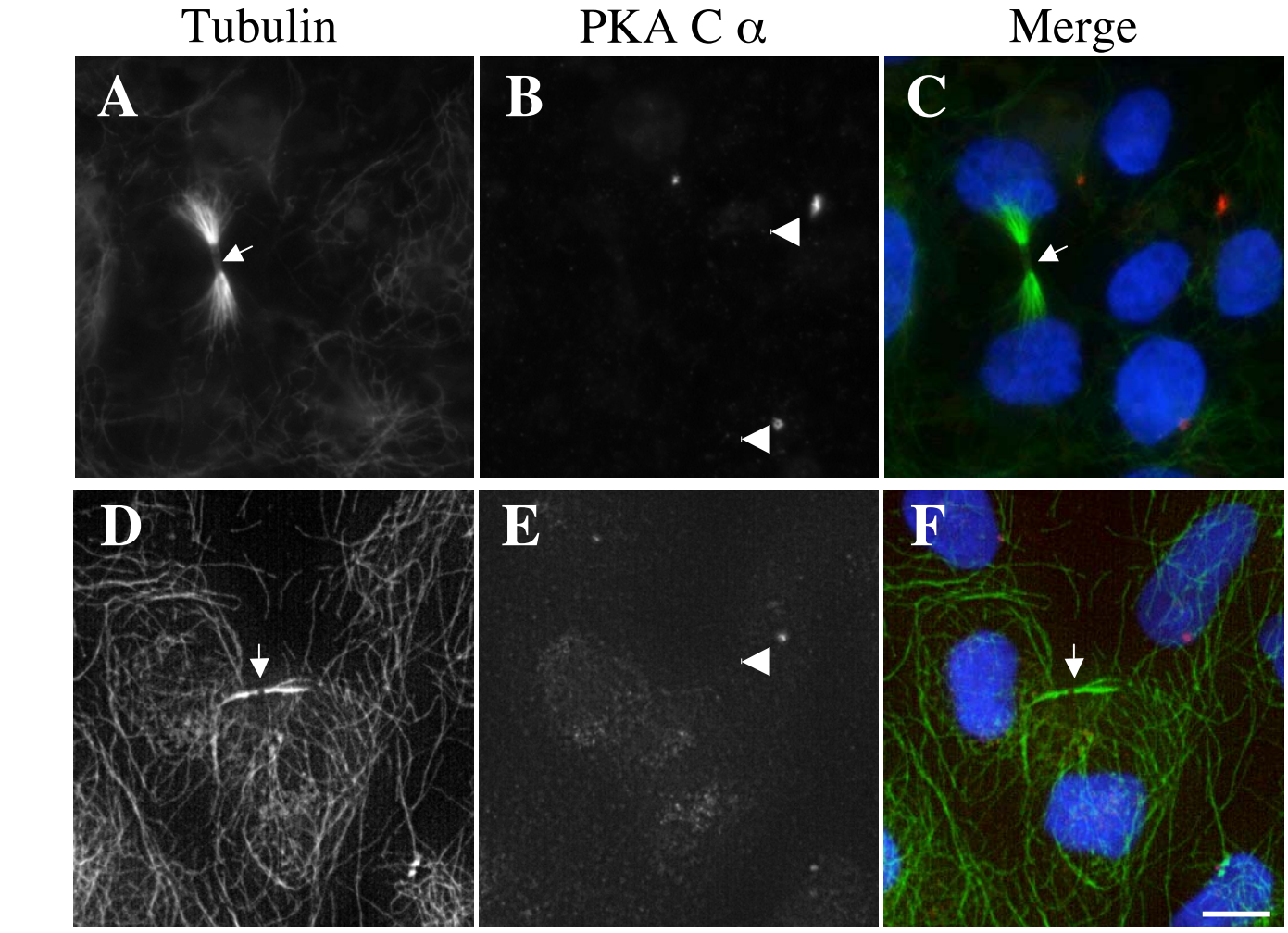


Fig 4.(continued): Quantification of Ht31GFP overexpressing HEK 293 cells show defective cytokinesis. (D-E) Shows the quantitation of the time-lapse imaging results. Vertical bars represent recording from single cells. Results represent recordings of individual cells from three to four independent experiments. (F-G) Presents the quantitation of the time-lapse imaging results of GFP-Akt expressing COS cells which showed defective cytokinesis.



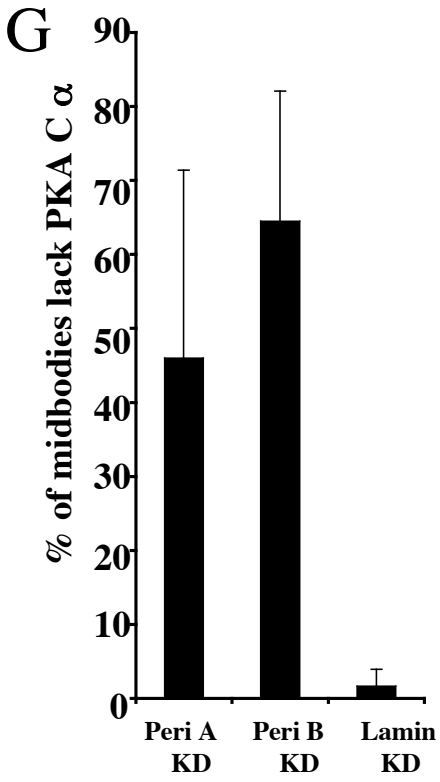


Fig 5. siRNA-depletion of pericentrin isoforms (A&B) results in mislocalization of PKA C α from the midbody. Micrograph shows the staining of U2OS cells treated with kendrin siRNA. Stained with PKA C α (red, B, E), tubulin (green, A, D) and DAPI (merge, C, F). Bar in F, 10 μm for A-F. Panel A-C are two dimensional images. Panel D-F are three dimensional images deconvolved and projected as two dimensional images to reveal all the stained material. Arrows in the images (A, C, D and F) shows the midbody region. Arrowheads in (B and E) show the centrosomal staining of PKA C α. (G) Shows the quantitation of the pericentrin isoforms abrogated telophase cell midbodies which lack PKA C α localization. Each bar represents the average of two experiments. In each experiment about 25-50 midbodies were counted per sample.

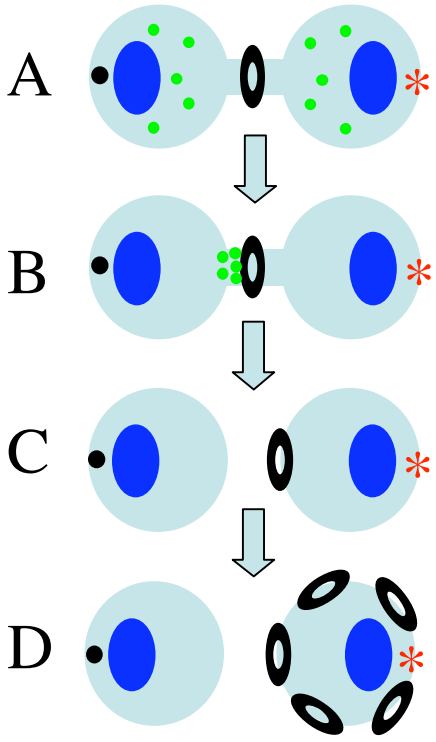


Figure 6. Asymmetric cell division in tumor cells. During human cell division, two replicated sets of chromosomes (DNA, blue, Fig. 1) must be segregated to opposite ends of the cell. The cell must then be severed to create two new progeny. ***In our recent studies, we unexpectedly uncovered five novel asymmetric events that cooperated to generate daughter cells that are not equal (Gromely et al).*** 1) In the first step, membrane vesicles (green dots, Fig. 1) move asymmetrically to *one side* of the compact midbody (MB, black ring, Fig. 1A, B). 2) The vesicles fuse coordinately with the cell membrane and with one another to sever the cell in two (Fig. 1B, C). This defines a new role for vesicle fusion in human cell division. 3) The midbody moves to the cell opposite the severing site (Fig. 1C). 4) The cell with this midbody derivative (MBd) also contains the original centriole (Fig. 1C, red asterisk), a structure that replicates once every cell division cycle and contributes to mitotic spindle formation and chromosome segregation. 5) Remarkably, in subsequent cell divisions MBds *accumulate* in this same cell (Fig. 1D). Thus, one cell receives MBds and the original centriole, whereas the other receives no MBds and a centriole copy. To our knowledge, there is no other example in cell biology where a singular organelle is asymmetrically inherited by one of the two daughter cells. Our most recent results demonstrate that prostate cancer cells (e.g. PC3) and other cancer cells-but not normal cells-accumulate midbodies (Table 1).

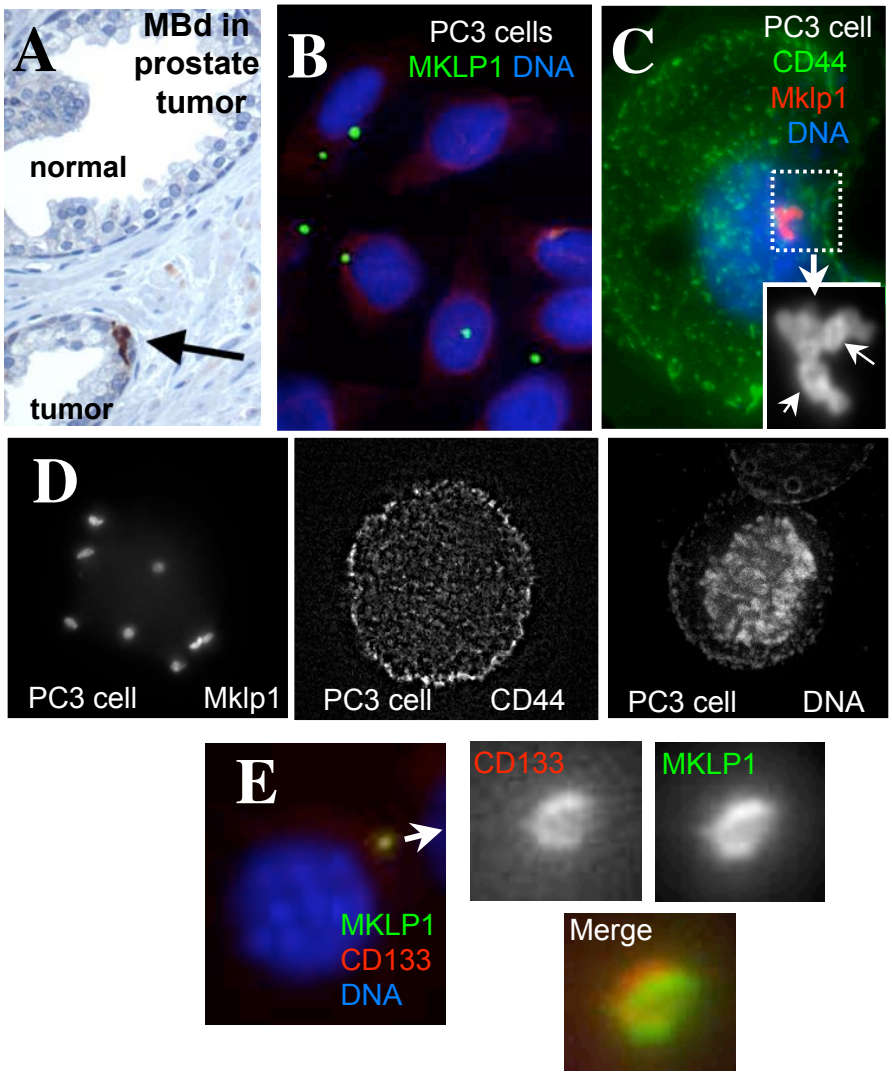


Figure 7. Midbody derivatives are in a subset of prostate cancer cells (putative cancer 'stem cells'?). A. Paraffin sections of prostate tumor showing rare prostate tumor cells stained for midbody proteins by immunohistochemical methods (MKLP1). Note 1: MKLP1 positive cells were often adjacent to the basal layer in early lesions, the position of putative prostate cancer stem cells. Note 2: Most other cells in the gland did *not* stain for midbodies. B. Midbodies were also present in many cells of the prostate cancer cell line PC3. C, D. Midbody-containing PC3 cells appeared to be putative prostate cancer stem cells as they stained for the presumed prostate cancer stem cell markers, CD44 (C) and CD133 (E). Note: CD133 localized to midbodies in PC3 cells (E) suggesting a possible function for the protein at this site. MBds did *not* localize to normal primary prostate epithelial cells (not shown). This data suggests that MBd-containing cells may be prostate CSCs.

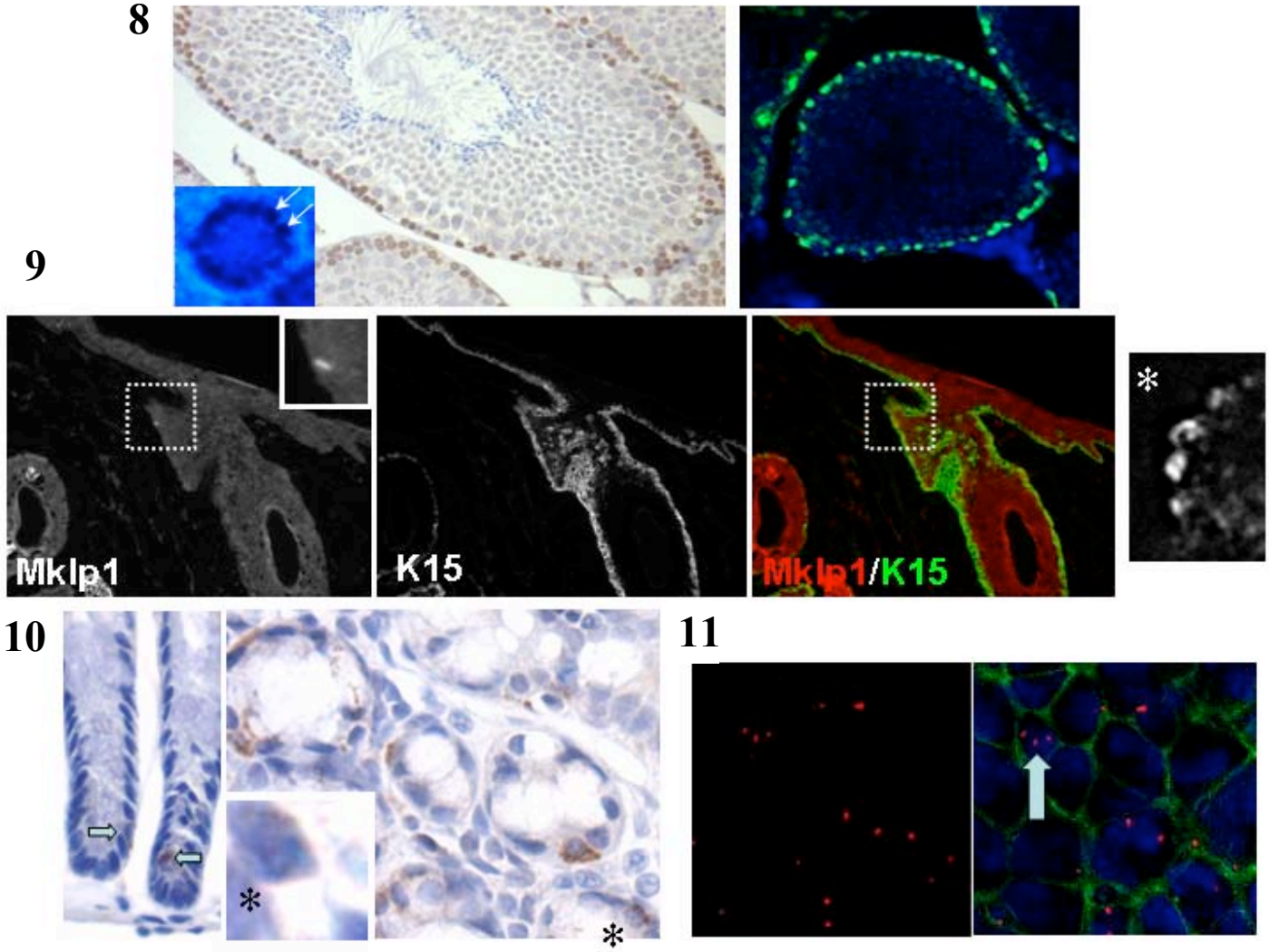


Figure legend. Fig. 8. Mouse testes histological section stained for MB^{d}s (left, brown color) and counterstained with hematoxylin (blue). Staining is largely confined to the basal cell layer (stem & pr ogenitor cells). Enlargement (bottom left) shows that staining in this individual cell is punctate, as expected for the presence of MB^{d}s. At right is testes stained for a stem cell marker (Oct 4) showing a similar pattern to MB^{d}s stain. **Fig. 9.** A section through a hair follicle (left, box enlarged upper right) shows that a small subset of cells that are positive for the epithelial marker K15 (middle, marks the stem cell compartment) stain for MKLP1 (MB^{d}s). Right, color merge. Far right image (*) shows ring-like structures stained with MKLP1 in another skin section (immunofluorescence)**. Fig. 10.** Section of mouse colon showing cells in a plane consistent with the +4/5 position (stem cells), stain for MB^{d}s. Inset, right higher magnification of cell at * showing multiple MB^{d}s. **Fig. 11** MB^{d}s (left) in H9 embryonic stem cells (right, merged with nuclei and actin). Arrow, cell with three MB^{d}s.

Table 1: MB derivatives are found in stem cells (and cancer 'stem cells'?)

(Prostate cancer results in red below)

Cell lines

Cell type	***(% cells, n>4000)***
hESCs	
H9	51.7
H9 after differentiation	4.6
NCCIT (hESC tumor)	66.8
Nonstem cells	
RPE1 (human)	0.9
Mouse tracheal cells	0.2
Mouse embryo fibroblasts	0.7
Cancer cell lines	
PC3	15.9
MDMB231	7.2
293T	29.1
HeLa	21.1

Are these prostate cancer stem cells?

Tissues

Tissue	***Location***
Prostate	rare cells near basal lamina
Prostate tumor	rare cells near basal lamina
Testes	stem cell compartment (MELK1+ cells)
Skin	stem cell compartment (bulge, K15+ cells)
Gut	stem cell compartment (+4/5 cells in crypts)
Breast	rare cells near basal lamina

Are MBds a universal marker for stem cells and cancer 'stem' cells/cancer 'initiating' cells?

KEY RESEARCH ACCOMPLISHMENTS

Task 1. Not initiated
9/11 disaster and fraud scandal led to termination of required collaborations.

Task 2.
a. Completed. Injection of pericentrin-expressing cells into mouse prostates does not enhance tumor growth
b. Partially completed, pericentrin PKA/B/C mutants were constructed and tested by transient transfection for genetic instability, but work was curtailed until we reconcile differences between the in vivo and in vitro results in Task 2a.
c, d. Curtailed. Work on pericentrin mutants will resume when we understand the result in Task 2a (above)
n.d. (new directions):
a'. Disruption of pericentrin's interaction with PKA, B or C induces aneuploidy (paper in preparation)
b'. Pericentrin anchors all kinases at the midbody to control cytokinesis (paper in preparation)
c'. Pericentrin interacting protein centriolin defines a new pathway for cytokinesis (Gromley et al, 2005)

Task 3.
a. Completed. Vectors have been constructed
b. Completed.
n.d. (new directions):
An offshoot of this work was the observation that midbodies accumulate in a subset of cells in prostate tumors and cell lines! We are testing if these are prostate cancer 'stem cells'/'cancer initiating cells' (see recent proposal submitted to the PCRP of the D.O.D. and reviews).

REPORTABLE OUTCOMES

- **Manuscripts:**

1. Gromley A, Yeaman C, Rosa J, Redick S, Chen C, Mirabelle S, Guha M, Sillibourne J, and Doxsey S. Centriolin Anchoring of Exocyst and SNARE Complexes at the Midbody Is Required for Secretory-Vesicle-Mediated Abscission. Cell, 123, 75-87, 2005.

2. Purohit A, Mikule K and Doxsey S. PKA and AKT are anchored at midbodies by pericentrin and control cytokinesis and maintain genetic stability. In preparation.

3. Chen C, Kuo T, Houghton J, Lyle S and Doxsey S. Midbodies accumulate in stem cells and possibly cancer stem/initiating cells. In preparation.

4. Baron D, Chen C, Kuo T and Doxsey S. Midbody-containing cancer cells have increased tumor-associated properties. In preparation.

♦ **Oral presentations by Stephen Doxsey:**

2003:
03/2003
Presenter: Stephen Doxsey (Plenary Lecturer)
Presentation title: "Emerging roles of centrosomes"
Meeting title: Conference of Union of the Swiss Societies for Experimental Biology
Meeting location: Davos, Switzerland.
03/2003
Presenter: Stephen Doxsey
Presentation title: "Centrosomes in division and disease"
Meeting title: Personal invitation, not meeting
Meeting location: Clark University, Department of Biology, Worcester, MA**.**
04/2003
Presenter: Stephen Doxsey
Presentation title: "Centrosomes in division and disease"
Meeting title: Personal invitation, not meeting
Meeting location: University of Pittsburgh, Department of Biological Sciences, Pittsburgh, PA.
05/2003
Presenter: Stephen Doxsey
Presentation title: "Centrosomes, aneuploidy and cancer"
Meeting title: Personal invitation, not meeting
Meeting location: Wistar Institute, University of Pennsylvania, Philadelphia, PA.
06/2003
Presenter: Stephen Doxsey
Presentation title: "Centrosome genes in checkpoint control, cell cycle progression and cytokinesis"
Meeting title: FASEB Summer Research Conference on Nuclear Structure and Cancer
Meeting location: Saxtons River, VT.
07/2003
Presenter: Stephen Doxsey
Presentation title: "Centrosome genes involved in cell cycle progression and genetic instability"
Meeting title: American Society for Cancer Research. RNAi: Opportunities and Challenges in Cancer Research
Meeting location: Washington, D.C.
07/2003
Presenter: Stephen Doxsey
Presentation title: "Emerging Roles of Centrosomes"
Meeting title: Conference: Marc-A-Thon (all 'scientific progeny' of Marc Kirschner)
Meeting location: Harvard Medical School, Boston, MA
09/2003
Presenter: Stephen Doxsey
Presentation title: "Mechanisms of Cell Division"
Meeting title: Conference of the Chilean Society of Biochemistry and Molecular Biology
Meeting location: Concepcion, Chile.
12/2003

Presenter: Stephen Doxsey
Presentation title: “siRNA-mediated centrosome damage activates a G1 checkpoint”
Meeting title: American Society for Cell Biology: Organelle Maintenance and Inheritance
Meeting location: San Francisco, CA

2004:
01/2004
Presenter: Stephen Doxsey
Presentation title: “Cytokinesis and aneuploidy”
Meeting title: Personal invitation, not meeting
Meeting location**:** Harvard University Department of Molecular Cell Biology, Cambridge, MA
01/2004
Presenter: Stephen Doxsey
Presentation title: “Centrosomes and aneuploidy in cancer”
Meeting title: Conference on Aneuploidy and Cancer, SICR
Meeting location: Oakland California.
01/2004
Presenter: Stephen Doxsey
Presentation title: “Centrosomes in division and disease”
Meeting title: Personal invitation, not meeting
Meeting location: UMass Medical School, Cancer Center, Worcester, MA.
01/2004
Presenter: Stephen Doxsey
Presentation title: “Centrosomes in division and disease”
Meeting title: Personal invitation, not meeting
Meeting location: Duke University, Department of Cell and Molecular Biology, Durham, NC.
02/2004
Presenter: Stephen Doxsey
Presentation title: “Centrosomes, aneuploidy and cancer”
Meeting title: Personal invitation, not meeting
Meeting location: Pfizer, Inc, Groton Research Laboratories, Groton, CT.
03/2004
Presenter: Stephen Doxsey
Presentation title: “Centrosomes, aneuploidy and disease”
Meeting title: Personal invitation, not meeting
Meeting location: National Cancer Institute, Microtubule and Mitosis Group of the Screening Technologies Branch, Washington, D.C.
03/2004
Presenter: Stephen Doxsey
Presentation title: “Emerging roles of centrosomes in cancer”
Meeting title: Personal invitation, not meeting
Meeting location: Tufts University School of Medicine, Dept of Anatomy and Cell Biology, Boston, MA.
03/2004
Presenter: Stephen Doxsey
Presentation title: “Emerging roles of centrosomes”
Meeting title: Personal invitation, not meeting
Meeting location: UMass Medical School, Department of Cell Biology, Worcester, MA.
06/2004

Presenter: Stephen Doxsey
Presentation title: "Centrioin-anchoring of exocyst and SNARE complexes at the midbody is required for localized secretion and abscission during cytokinesis"
Meeting title: Conference on Cytokinesis (ASCB Summer Meeting)
Meeting location: Burlington, VT

06/2004
Presenter: Stephen Doxsey
Presentation title: "Centrosome-mediated mechanisms of genetic instability"
Meeting title: Conference on Genomic Integrity in Cancer, General Motors Research Foundation
Meeting location: Washington, D.C.

09/2004
Presenter: Stephen Doxsey (Keynote Speaker)
Presentation title: "Emerging roles of centrosomes"
Meeting title: Conference on Highlights in Basic and Translational Cancer Research
Meeting location: Rotterdam, The Netherlands,

09/2004
Presenter: Stephen Doxsey
Presentation title: "Centrosome genes involved in cytokinesis and chromosome missegregation"
Meeting title: 6th International Workshop on Chromosome Segregation and Aneuploidy
Meeting location: Tuscany, Italy.

08/2004
Presenter: Stephen Doxsey
Presentation title: "Centrosomes and tumorigenesis"
Meeting title: Asia-Pacific Conference of Tumor Biology and Medicine
Meeting location: Xi'an, China.

10/2004
Presenter: Stephen Doxsey
Presentation title: "Emerging roles of centrosomes"
Meeting title: Personal invitation, not meeting
Meeting location: University of New Mexico, Dept Molecular Genetics & Microbiology, Albuquerque, NM.

2005:

01/05
Presenter: Stephen Doxsey
Presentation title: "Emerging roles of centrosomes"
Meeting title: Personal invitation, not meeting
Meeting location: University of Connecticut Health Sciences, Dept. Medicine, Framingham, CT

02/05
Presenter: Stephen Doxsey
Presentation title: "Mitosis and human cancer"
Meeting title: Personal invitation, not meeting
Meeting location: Assumption College, Worcester, MA

02/05
Presenter: Stephen Doxsey
Presentation title: "Imaging centrosomes in cancer"
Meeting title: Live Cell Imaging Conference
Meeting location: Hong Kong, China

10/2005

Presenter: Stephen Doxsey
Presentation title: “Prognostic potential of centrosome defects in cancer”
Meeting title: UMass: Innovation Partner for the Medical Device Industry
Meeting location: Newton, MA
10/2005
Presenter: Stephen Doxsey
Presentation title: “Emerging roles of centrosomes in polycystic kidney disease”
Meeting title: Personal invitation, not meeting
Meeting location: Harvard Institutes of Medicine, Dept. Molecular and Developmental Genetics, Boston, MA
04/05
Presenter: Stephen Doxsey
Presentation title: “Role of centrosomes in cancer”
Meeting title: Personal invitation, not meeting
Meeting location: Cytyc Corporation, Inc, Marlboro, MA
06/05
Presenter: Stephen Doxsey
Presentation title: “Emerging roles of centrosomes in biology and disease”
Meeting title: Personal invitation, not meeting
Meeting location: Burnham Institute, La Jolla, CA
06/05
Presenter: Stephen Doxsey
Presentation title: “Centrosomes and aneuploidy”
Meeting title: FASEB meeting on nuclear structure and cancer.
Meeting location: Saxtons River, VT
06/05
Presenter: Stephen Doxsey
Presentation title: “Centrosomes and cytokinesis”
Meeting title: *Gordon Research Conference: Cell Motility and the Cytoskeleton*
Meeting location: Colby-Sawyer College, NH
07/05
Presenter: Stephen Doxsey
Presentation title: “Centrosomes, membrane traffic and cytokinesis”
Meeting title: Personal invitation, not meeting
Meeting location: Burnham Institute, La Jolla, CA
09/05
Presenter: Stephen Doxsey (Co-organizer)
Presentation title: “Centrosomes, membrane traffic and cytokinesis”
Meeting title: Centrosome and spindle poles Conference
Meeting location: Heidelberg, Germany
11/05
Presenter: Stephen Doxsey
Presentation title: “A role for centrosomes in polycystic kidney disease”
Meeting title: American Society of Nephrology
Meeting location: Philadelphia, PA
11/05
Presenter: Stephen Doxsey (Co-organizer)
Presentation title: “Asymmetries during cell division: roles in stem cells and aging”
Meeting title: UMass Research Retreat
Meeting location: Woods Hole, MA

2006:
02/06
Presenter: Stephen Doxsey
Presentation title: "Asymmetries during cell division: roles in stem cells and aging"
Meeting title: Personal invitation, not meeting
Meeting location: Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany
03/06
Presenter: Stephen Doxsey
Presentation title: "Asymmetries during cell division: roles in stem cells and aging"
Meeting title: Personal invitation, not meeting
Meeting location: University of Texas South Western, Dallas, TX
04/06
Presenter: Stephen Doxsey
Presentation title: "Centrosomes and cancer"
Meeting title: Personal invitation, not meeting
Meeting location: The Norwegian Radium Hospital, Institute for Cancer Research, Oslo, Norway
04/06
Presenter: Stephen Doxsey
Presentation title: "Asymmetries during cell division: are daughter cells born equal?"
Meeting title: Personal invitation, not meeting
Meeting location: Oregon Health Science Center, Portland, OR
04/06
Presenter: Stephen Doxsey
Presentation title: "Asymmetries during cell division and their consequences"
Meeting title: Personal invitation, not meeting
Meeting location: University of Oklahoma Health Science Center, Oklahoma City, OK
05/06
Presenter: Stephen Doxsey
Presentation title: "Asymmetries during cell division and their consequences"
Meeting title: Personal invitation, not meeting
Meeting location: Curie Institute, Research Section, Paris, France
06/06
Presenter: Stephen Doxsey
Presentation title: "Asymmetries during cell division and their consequences"
Meeting title: Personal invitation, not meeting
Meeting location: Columbia University, New York, NY
06/06
Presenter: Stephen Doxsey
Presentation title: "Centrosomes, aneuploidy and cancer"
Meeting title: Personal invitation, not meeting
Meeting location: Astra Zeneca, Waltham, MA
07/06
Presenter: Stephen Doxsey
Presentation title: "Asymmetries during cell division and their consequences"
Meeting title: Personal invitation, not meeting
Meeting location: Swiss Federal Institute of Technology, Zurich, Switzerland
11/06

Presenter: Stephen Doxsey
Presentation title: "Asymmetries during cell division and their consequences"
Meeting title: Personal invitation, not meeting
Meeting location: Wadsworth Center, Albany New York

- **Patents.** Licensed two patents to Cytyc, Inc. for cancer detection and prognosis: *Cancer Detection by Centrosome Abnormality* (#5,972,626) and *Cancer Prognosis by Centrosome Detection*

- **Development of permanent cell lines:** Developed cell lines expressing GFP-centrin2, GFP-gamma tubulin, GFP-GapCenA, RFP-signal peptide; Centriolin, centriolin C terminus, GFP-MKLP1 PC3 cells under construction.

- **Work that started as a new direction in this proposal** was recently submitted to the PRCP of the D.O.D. It received a score of Excellent but was not funded (see PC073330 and reviews in appendix). A proposal focused on breast cancer stem cells and midbodies was sent to the BCRP received a score of Outstanding and was recently funded and chosen to be highlighted in the D.O.D. annual report (BC074714).

- **Institutional and departmental support based on research supported by this award.** The P.I. (SJD) was given departmental and institutional funds ($240,000) to purchase a spinning disc confocal microscope to continue work on cytokinesis and aneuploidy related to this project.

- **Institutional and departmental support based on research supported by this award.** Based on work related to this project on cytokinesis, aneuploidy and stem cells, the P.I. (SJD) was chosen to apply for a highly competitive W.M. Keck Foundation proposal. This application was funded.

- **Engaged in sponsored research agreement with Cytyc, Inc. (2005-2007)** to determine if centrosome defects are a prognostic indicator of aggressive cervical cancer.

- **Engaged in sponsored research with AstraZeneca Inc. (2006-2008)** to test anti-cancer drugs in the clinic for effects on mitosis and cytokinesis.

- **Other Relevant Items (News articles):**
  Media 1: Article discussing our paper showing that centriolin depletion induces cytokinesis failure and aneuploidy--appeared in the Telegram & Gazette (Worcester, 10/10/05)
  Media 2: Article in Boston Globe on same study as above (10/10/05.
  Media 3: Article in Focus (UMass publication) on above (11/05)

CONCLUSIONS

We believe that our work on centrosome dysfunction will have a significant impact on our understanding of prostate cancer progression, the etiology of prostate cancer and treatment of the more aggressive and devastating forms of this disease. Insights gained from this approach should yield novel information on the mechanisms (centrosomes, cytokinesis) and molecules (pericentrin, centriolin, Akt, PKA, PKC) that contribute to genetic instability and prostate cancer development and progression. The identification of a new pathway involved in cell division through vesicle-mediated abscission (cell separation) provides a new piece of biology to investigate in search for therapeutic targets that are altered by mitotic drugs (e.g. taxanes). The numerous asymmetric events that we identified during mitosis (vesicle traffic, older centrosome, midbody inheritance) that culminate in the creation of two *different, not identical* daughter cells, offer new pathways to study in the context of normal and cancer cell division. The identification of midbodies in prostate cancer 'initiating/stem

cells' has important implications for prostate cancer stem cell identification and cell-specific targeting of prostate cancer. It is our belief that these putative prostate cancer stem cells should be the focus of therapies as it is these cells that appear to persist when all other cells are killed by chemotherapy in vitro (Desiree Baron, Steve Doxsey, personal communication). The midbody itself--being a key player in the late stages of mitosis--has become an important focus of recent research (our paper by Gromley et al, 2005 has been cited over 200 times) and we believe that it could serve as a target for prostate cancer therapies. We hope to develop a company around the potential of post-mitotic midbodies to serve as stem cell and cancer stem cell markers to retrieve such cells from different organs and tumors for further study. In future studies, we hope to test the hypothesis that post-mitotic midbodies contribute to stem cell/cancer stem cell function.

REFERENCES

Chen, D., Purohit, A., Halilovic E, Doxsey S, Newton, A. Protein kinase C anchoring at the centrosome controls cytokinesis. *Authors contributed equally. J. Biol. Chem., 279, 4829, 2004 ***(with cover photo).***

Doxsey, S., McCollum, D. & Theurkauf, W. Centrosomes in cellular regulation. *Annu Rev Cell Dev Biol* 21, 411-434 (2005a).

Doxsey, S., Zimmerman, W. & Mikule, K. Centrosome control of the cell cycle. *Trends Cell Biol* 15, 303-311 (2005b).

Gromley A, Yeaman C, Rosa J, Redick S, Chen C, Mirabelle S, Guha M, Sillibourne J, and Doxsey S. Centriolin Anchoring of Exocyst and SNARE Complexes at the Midbody Is Required for Secretory-Vesicle-Mediated Abscission. Cell, 123, 75-87, 2005. *(see reviews in Dev. Cell by B. Bement, Curr Biol by D. McCollum and J Cell Biol)*

Lingle WL, Barrett SL, Negron VC, D'Assoro AB, Boeneman K, Liu W, Whitehead CM, Reynolds C, Salisbury JL. Centrosome amplification drives chromosomal instability in breast tumor development. Proc Natl Acad Sci U S A. 2002 Feb 19;99(4):1978-83.

Mikule K, Delaval B, Kaldis P, Jurcyzk A, Hergert P, Doxsey S. Loss of centrosome integrity induces p38/p53/p21-dependent G_1/S arrest. Nature Cell Biol, 9, 160-170, 2007.

Pihan, G. et al. Centrosome defects and genetic instability in malignant tumors. *Cancer Research* 58, 3974-3985 (1998).

Pihan, G. A. et al. Centrosome defects can account for cellular and genetic changes that characterize prostate cancer progression. *Cancer Res* 61, 2212-9. (2001).

Pihan, G. A., Wallace, J., Zhou, Y. & Doxsey, S. J. Centrosome abnormalities and chromosome instability occur together in pre-invasive carcinomas. *Cancer Res* 63, 1398-404 (2003).

Pihan, G. and Doxsey, S. Mutations and aneuploidy: Co-conspirators in cancer? Cancer Cell 4, 89-94, 2003.

Bibliography (publications supported in part by this proposal):

Gromley A, Yeaman C, Rosa J, Redick S, Chen C, Mirabelle S, Guha M, Sillibourne J, and Doxsey S. Centriolin Anchoring of Exocyst and SNARE Complexes at the Midbody Is Required for Secretory-Vesicle-Mediated Abscission. Cell, 123, 75-87, 2005.

Aruna Purohit, Keith Mikule and Stephen Doxsey. PKA and AKT are anchored at midbodies by pericentrin and control cytokinesis and maintain genetic stability. In preparation.

Chun-Ting Chen, Tse-Chun Kuo, JeanMarie Houghton, Steve Lyle and Stephen Doxsey. Midbodies accumulate in stem cells and possibly cancer stem/initiating cells. In preparation.

Desiree Baron, Chun-Ting Chen, Tse-Chun Kuo and Stephen Doxsey. Enriched fractions of midbody-containing cancer cells have increased tumorigenic properties. In preparation.

DEPARTMENT OF DEFENSE
U.S. ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
CONGRESSIONALLY DIRECTED MEDICAL RESEARCH PROGRAMS

2007 PROSTATE CANCER RESEARCH PROGRAM

PEER REVIEW PANEL SUMMARY STATEMENT
(PRIVILEGED COMMUNICATION)

Proposal Number:	PC073330
Grants.gov ID Number:	GRANT00263277
Award Mechanism:	Idea Development Award
Review Panel:	Cell Biology #1
Meeting Dates:	07/11/2007–07/13/2007
Principal Investigator:	Stephen Doxsey
Contracting Organization:	Massachusetts, University of, Medical School
Performing Organization:	Massachusetts, University of, Medical School
Project Title:	A Novel Prostate Cancer Target, the Midbody, a Complex Singular Organelle, Created during Cell Division, That Is Asymmetrically Inherited by Cancer Stem Cells
Score:	2.0
Standard Deviation:	0.12
Proposed Start Date:	09/30/2008
Proposed End Date:	09/29/2011
Total Budget Requested:	$609,375
Direct Cost:	$375,000
Indirect Cost:	$234,375

SUMMARY:

The Principal Investigator (PI) on this proposal for an Idea Development Award is basing his work on the finding that prostate cancer cells (PC3 and DU-145) undergo asymmetric cell division that generates non-identical daughter cells, such that one daughter cell inherits the midbody (MB) and the other daughter cell inherits the post-mitotic MB, called the midbody derivative (MBd). The hypothesis to be tested is that MBds may serve as markers for prostate cancer stem cells and may have diagnostic/prognostic value for prostate cancer progression. Personnel constitute a major **strength**. The PI is an accomplished investigator in the proposed area of research with a record of publication in superior journals. He has assembled a highly qualified research team. In addition, the idea that MBds may serve as putative prostate cancer stem cell markers is original and novel. If the PI is able to successfully link MBds to prostate cancer stem cells, this work will have an impact on the field in terms of basic biological studies. There are some **weaknesses**. The underlying concept is now accepted by the general scientific community and thus is no longer as novel as it once was. For some experiments, there may be problems with feasibility and data interpretation. It is unlikely that this work will have an impact on prostate cancer therapy in the near future. Overall, the proposal is rated **Excellent**; although

an immediate effect on prostate cancer therapy is unlikely, the work will probably have an impact on basic biological research in prostate cancer. The panel reviewed the proposal on the basis of the published evaluation criteria and rated it as described below.

Evaluation Criteria Rating Scale 1 (low merit) to 10 (high merit):

Criteria Description	Score
Innovation	6.8
Impact	7.2
Research Strategy	5.6
Personnel	9.0
Environment	8.6

EVALUATION CRITERIA:

Innovation:

Scientist Reviewer A: The finding that two prostate cancer cells resulting from a cell division are different, with one daughter cell inheriting the midbody, is fundamentally important. The idea that MBd can serve as putative prostate cancer stem cell markers is original. The potential to target MBd for prostate cancer therapy in future studies also represents a new concept.

Scientist Reviewer B: Work from this laboratory is innovative in that the investigators are the ones that previously discovered the disproportionate allocation of the MB. They have carried out studies further characterizing this phenomenon to determine if it is an indicator of cancer stem cell identity. The work that they propose, which follows along with their earlier studies, is designed to strengthen this innovative aspect with the exception that the work in itself is now more accepted by the general scientific community and thus is no longer as novel as it once was. It is possible that MBs can be a therapeutic target in prostate cancer. However, this is the weakest prospect of the innovation characteristic.

Panel Comments: The PI has been following up on his initial observation for some time and the concepts to be tested are not new to this proposal.

Impact:

Scientist Reviewer A: The proposed research will address the significance of asymmetric division of prostate cancer cells, which is fundamentally important. The proposed studies will define the role of MBd in prostate cancer progression, which may uncover novel and previously unexplored mechanisms underlining prostate cancer etiology. The success of the proposed study may define MBd as a new prostate cancer stem cell marker, providing the foundation for future

studies to test whether the MBd proteins can be used as a prognostic and/or diagnostic marker. Furthermore, MBds may represent a novel therapeutic target for prostate cancer prevention or elimination.

However, since the applicant has not co-stained for prostate stem cell markers with markers for MBd, it is somewhat speculative that MBd will serve as a marker for prostate cancer stem cells. As MBd is rarely detected in prostate cancer specimens, it could be a challenge to use MBd as a marker for diagnosis and prognosis.

Scientist Reviewer B: Although there is no doubt that cancer stem cells are likely responsible for the generation of many cancers, the identification of stem cells and studying them in the context of *in vivo* biology has been difficult. Although there are many markers for cancer stem cells that have been developed over time, the development of the MB assay from this laboratory—if in fact MBds are directly linked to stem cells—will have a significant impact on the field in basic biological studies and potentially down the road in therapy, although at the moment, this is a long shot. If the PI successfully links MBds to prostate cancer stem cells, however, there is no doubt that this work will impact the field.

Research Strategy:

Scientist Reviewer A: Specific Aim 1 will determine if MBds are present in prostate stem cancer cells using putative stem cell markers. Cultured human prostate cancer cell lines, cells disassociated from human prostate cancer, and tissue sections containing prostate cancer will be used in this study. The PI has expertise in the proposed research and should have no problem in conducting it. The weakness is that the number of prostate tumor specimens, their clinical stage, and Gleason grade were not indicated. No contingency and alternative is provided if MBds are not colocalized with putative stem cell markers.

The PI also proposed to determine if MBds are present in prostate cancer side populations, rare populations express the multidrug resistance transport on their cell surface for active efflux of the Hoechst 33342 dye. The applicant indicated in Aim 1b that "side populations have already been isolated from PC3, LNCaP and DU-145 cell lines and have been shown to be tumorigenic" citing a reference. However, according to a table in that same reference, side population is not detectable in PC3, DU-145, and LNCaP cells. Only LAPC9 had 0.07 percent side population in five different assayed prostate tumor cells in the reference. Thus, MBds are not present in prostate cancer side populations. This also casts doubt on whether MBd can serve as a marker for prostate cancer stem cells. It is not clear if MBds can be detected in LNCaP cells.

The PI also proposes to determine if MBds are in prostate cancer cells that retain the BrdU for long periods of time. The PI should have the expertise to conduct the proposed research, if the prostate cancer cell lines contain the label-retaining cells.

In Specific Aim 2, the PI will isolate and test MBd-containing prostate cancer cells for cancer stem cell properties. Given their experience using HeLa cell line, the proposed experiments to construct and select PC3 cell lines expressing GFP-MKLP-1 or GFP-Cep55, two markers for MBds, are feasible. Results of tests of MBd+ and MBd– cells for their ability to form tumors in

mice and grow in soft agar will determine if MBd is associated with aggressiveness of cancer cells.

In Specific Aim 3, the PI will further test whether MBd disruption alters the tumor potential of prostate cancer cells using either microtubular depolymerizing agent nocodazole or RNAi-mediated depletion of MBd proteins. This experiment is feasible using PC3 and DU-145 because they have MBd. However, it is not clear whether LNCaP cells or primary prostate cancer cells from human and mouse tumor will have sufficient numbers of cells positive with MBd. Thus, it is not clear if the proposed MBd disruption experiments will be feasible in these cells. A potential complication is that nocodazole or RNAi will affect cellular components other than MBd, which could make data interpretation difficult.

Scientist Reviewer B: Following the initial discovery of the disproportionate allocation of the MB in cell division of cancer cells by this laboratory, further studies have developed in both prostate and other cancers and rightfully shown that MBds may be associated with cancer stem cells. Their hypothesis for the current application is that the MBds will serve as markers for prostate cancer stem cells that will have diagnostic and prognostic value for prostate cancer progression. Although this is a laudable hypothesis, what is actually proposed does not fully address this.

There are three specific aims. The first aim is to link MB to prostate cancer stem cells. The second aim is to isolate and test whether prostate cancer stem cells have increased capability to form tumors *in vivo*. The third aim is to test whether MB disruption alters the tumorigenic potential of prostate cancer cells. All of this work can be carried out by this laboratory and as proposed, will address the issue of use as a diagnostic. The third aim is very important, but its translation to clinical employment is long in the future.

An important strength of the proposal includes determining if MBds are located in a higher percentage of side population cells. A proposed experiment of interest will be to see if MBd cells have a higher capability, both *in vitro* and *in vivo*, to form tumors. A minor concern in this aspect is that the quantity of the side population fraction in the three tumor cell lines is rather limited, and whether or not they will be able to obtain sufficient quantities of cells to study, particularly in the case of LNCaP, may be problematic. It is likely that isolation of side populations will be problematic based on the PI's literature references.

Aim 2 is to isolate and test MB-containing cells for their cancer forming properties. Again, this work can be carried out with the above-noted caveat. They have two markers that seem useful to identify MBs. The proposal, as described, should allow them to identify and collect GFP-positive MB cells. A minor note here is that throughout the proposal the word "xenograph" is used when in reality the word is "xenograft."

Specific Aim 3 will be to examine how this population of cells behaves if the MB is disrupted. This aim is dependent upon the identification of MBds as being clearly associated with cancer stem cells, work that is being carried out in Aims 1 and 2. It is difficult to predict what the results of these studies will be. This latter part of Specific Aim 3 is problematic.

Personnel:

Scientist Reviewer A: The PI is a professor in the Program of Molecular Medicine at the University of Massachusetts Medical Center. Dr. Doxsey received a B.A. in biology from the University of Connecticut (1977) and a Ph.D. in cell biology from Yale University in 1987. Following postdoctoral training at the University of California at San Francisco, he joined the faculty at the University of Massachusetts Medical Center. The PI has published in excellent journals and has made significant contributions in the field of proposed research. He has also recruited Dr. Jean Marie Houghton, an expert in stem cell biology; Dr. Stephen Lyle, Director of Human Cancer Tissue Bank, University of Massachusetts; and Dr. Dario Altieri, an accomplished researcher with expertise in mouse tumor models. Clearly, the PI and the research team are qualified to conduct the proposed research.

Scientist Reviewer B: The personnel on this project are all well qualified and the PI meets the eligibility requirements. The background of the team and their respected expertise is without question. It would appear that the level of effort is appropriate for successful conduct of the proposed work.

Environment:

Scientist Reviewer A: The University of Massachusetts Medical Center has all the necessary facilities for the proposed studies. A concern is that the PI did not indicate whether he would have assistance from a pathologist specializing in prostate cancer.

Scientist Reviewer B: The environment is highly regarded for the accomplishment of the specific aims as proposed. Some of this research originated at this institution in the PI's laboratory. The facilities and resources are excellent. The quality and extent of institutional support is very strong.

Budget:

The budget as requested is appropriate in amount and duration. Final budget is subject to Programmatic Review and negotiations.

TECHNICAL ABSTRACT

Background. Prostate carcinoma is the most prevalent gender-specific cancer in the USA. Our previous D.O.D.-funded work on prostate cancer focused on defects during mitosis that induced genetic instability in prostate carcinoma and pre-invasive lesions (Pihan et al, 1998, 99, 01, 03a, b). In continuing studies, we unexpectedly found that prostate cancer cells (PC3, DU-145) and others (HeLa, U2OS) experienced a series of asymmetric events during cell division that generated *different* daughter cells (Gromley et al, Cell, 2005) and not identical cells as previously believed. Remarkably, one of the two daughter cells inherited the midbody (MB), a large organelle that assembles between the two dividing daughter cells. We call the post-mitotic MB inherited by one daughter, the midbody derivative (MBd). Remarkably, the cell with the MBd accumulates them with each subsequent cell division. This novel finding changes our perception of the fundamental process of cell division.

Preliminary results. To learn about the functional significance of MBds, we examined MBds in human and mouse prostate tumors and glands. In tumor sections, rare cells stained for MBds. These cells were sometimes adjacent to the basal layer, the position of putative prostate cancer stem cells (CSCs). Many cells in the prostate cancer cell line PC3, contained MBds, which stained for the putative prostate CSC markers, CD133 and CD44; CD13 was on MBds suggesting a role at this site. MBds were not found in progenitor cells, dividing non-stem cells or differentiating cells. This suggests that cells with MBds are putative prostate CSCs. Our work is consistent with the emerging view that prostate cancer develops from transformation of stem cells, which divide to produce a stem cell and a cell that ultimately differentiates. The definitive prostate CSC has not yet been identified so we asked if MBds were present in stem cells in tissues. MBds were present in stem cell compartments in the testes (basal layer, Oct4+/MELK+), hair follicle (a subset of K15+ cells), gut (+4/5 position from bottom of crypt) and brain (subventricular zone, radial glia/astrocytes, GFAP+, nestin-). MBds were found in human embryonic stem cells (H9), which were dramatically reduced upon differentiation. Dividing hepatocytes in regenerating liver, activated T cells and transit amplifying cells in all organs, above lacked MBds. These results suggest that MBds are in normal stem cells and prostate CSCs in vivo and in vitro.

Hypothesis. We propose that MBds will serve as markers for prostate CSCs, may have diagnostic/prognostic value for prostate cancer progression could directly contribute to prostate carcinoma.

Specific aims/Study Design: ***Aim 1.*** Determine if MBds are present in prostate CSCs in tumors and cell lines. a) Determine if co-stain with putative stem cell markers. b) Determine if MBds are in side populations of prostate cancer cells. c) Determine if MBds are in prostate cancer cells that retain BrdU (label retaining cells). ***Aim 2.*** Isolate and test MBd-containing prostate cancer cells for CSC properties. Human prostate cancer cell lines (initially PC3) expressing the MBd marker GFP-MKLP1 will be used to isolate MBd+ cells by flow cytometry based on object (MBd) size and intensity. MBd+ and MBd- cells will be tested for their ability to form tumors in mice and for transformation properties in vitro. ***Aim 3.*** To test if loss of MBd inheritance in prostate CSCs causes loss of stem cell properties, induction of differentiation and death/senescence. a) MBd inheritance will be disrupted in PC3 cell lines by randomizing MBd inheritance before division by microtubule depolymerization or by eliminating MBds by RNA interference of MBd genes. b) MBds will be eliminated in cells dissociated from human tumors using similar strategies.

Innovation and Impact. Innovative aspects of this proposal are: 1) the unexpected observation that cell division creates two dramatically different daughter cells, 2) that a complex organelle (MBd) is inherited by and accumulates in putative prostate cancer stem cells, 3) that the MBd could serve as a prostate cancer stem cell marker, 4) that MBds could serve as prognostic indicators of cancer progression, 5) that MBds in putative prostate cancer stem cells could contribute to prostate cancer etiology and 6) that MBds could become a viable target for prostate cancer treatment.

Research Team. We have amassed a group of experts in cancer biology that collectively have all the required expertise to complete this project (techniques, cells, animal protocols, prostate cancer experience).

PUBLIC ABSTRACT

Background. Prostate carcinoma is the most prevalent gender-specific cancer in the USA. Our previous D.O.D.-funded work on prostate cancer focused on defects during cell division that induced genetic (DNA) changes in prostate cancer and early prostate lesions (Pihan et al, 1998, 1999, 2001, 2003a, b). In continuing studies on this topic, we unexpectedly found that prostate cancer cells and others experienced a series of asymmetric events during the final stage of cell division that created two daughter cells that were *different* (Gromley et al, Cell, 2005), rather than creating two identical cells as previous studies had suggested. Only one of the two daughter cells received the midbody (MB), a large *singular* structure created during cell division. This observation reverses the notion that cells are created equal and changes the way we think about the fundamental process of cell division. We call the post-mitotic MB that is inherited by one daughter cell, the midbody derivative (MBd). Remarkably, the cell that receives the MBd accumulates additional MBds during subsequent cell divisions.

Preliminary results. To learn more about the inheritance and function of MBds, we examined MBds in human and mouse prostate tumors and glands. We identified a small fraction of tumor cells that had MBds. These cells were in positions occupied by putative prostate cancer stem cells (CSCs) and they often stained with putative prostate CSC markers. These observations are consistent with the emerging view that prostate cancer develops from malignant transformation of stem cells. Cancer stems, like normal stem cells, divide to produce another stem cell (a reproduction of itself) and a cell that ultimately acquires the characteristics of the prostate gland (through differentiation). Importantly, MBds were *not* found in non-stem cells and thus appeared to be selective if not specific for CSCs.

The definitive prostate CSC has not yet been identified. For this reason, we tested for the presence of MBds in well-documented stem cells. We found them in the appropriate positions in several human and mouse tissues (testes, hair follicle, gut and brain. In tissues for which stem cell markers were available, we found co-localization of MBds and markers. These and other results suggested that MBds were in normal stem cells and prostate CSCs in vivo and in vitro.

Specific aims: ***Aim 1.*** We will perform additional studies to confirm and extend the observation that MBds are present in prostate CSC compartments in both human prostate cancer cell lines and human prostate tumors. We will use all available CSC markers and other reliable methods to identify stem cells. ***Aim 2.*** We will test if MBd-containing prostate cancer cells have CSC properties when compared to MBd-negative prostate cancer cells. We will isolate MBd-positive cells from human tumors and test them for their ability to form tumors in mice and for tumor transformation properties in vitro. They will be compared to MBd-negative cells. ***Aim 3.*** To test if loss of MBds from prostate CSC induces loss of stem cell properties, differentiation and death. To accomplish this we will disrupt MBd inheritance or eliminate MBds in prostate cancer cells and in prostate tumors and test for tumor cell death and tumor regression or lack of formation.

Hypothesis. We propose that MBds will serve as valuable markers for prostate CSCs. They may also have diagnostic/prognostic value for prostate cancer progression. Finally, they we hypothesize that they directly contribute to prostate cancer development or progression.

Innovation and Impact. Innovative aspects of this proposal are: 1) that a complex organelle (MBd) is inherited by and accumulates in putative prostate cancer stem cells, 2) that the MBd could serve as a prostate cancer stem cell marker, 3) that MBds could serve as prognostic indicators of cancer progression, 4) that MBds in putative prostate cancer stem cells could contribute to prostate cancer progression and 5) that MBds could become a viable target for prostate cancer treatment.

Research Team. We have amassed a group of experts in cancer biology that collectively have all the required expertise to complete this project (techniques, cells, animal protocols, prostate cancer experience).

STATEMENT OF WORK

Task 1 (Aim 1): Determine if MBds are present in prostate CSCs.

a. Quantify the percentage of cells containing MBds and the number of MBds/cell in prostate cancer cell lines of different tumorigenic potential (PC3>DU-145>LNCaP, control, PrECs). Months 1-3

b. Quantify the percentage of cells containing MBds and the number of MBds/cell in prostate cancer cell lines of different tumorigenic potential (PC3>DU-145>LNCaP, control, PrECs) after isolating putative stem cells using markers (CD34, CD133, others). Months 3-6

c. Perform similar studies as in a and b using cells dissociated from human prostate tumors (12 tumors total). Months 6-12

d. Quantify the percentage of cells containing MBds and the number of MBds/cell in side populations (SP) of prostate cancer cell lines of different tumorigenic potential (PC3, DU-145, LNCaP, control, PrECs). Months 1-3

e. Quantify the percentage of cells containing MBds and the number of MBds/cell in side populations (SP) of prostate cancer cells dissociated from human tumors. Months 3-6

f. Determine if MBds are in prostate cancer cells that retain BrdU (label retaining cells, PC3, DU-145, LNCaP, control, PrECs). Months 1-3

g. Determine if MBds are in cells in xenografts of prostate cancer cells that retain BrdU (label retaining cells, PC3, DU-145, LNCaP, control, PrECs). Months 3-6

Task 2 (Aim 2): Isolate and test MBd-containing prostate cancer cells for CSC properties.

a. Generate GFP-tagged MKLP1 (MBd component) construct with antibiotic resistance (GFP-Cep55 is an alternative). Months 1-2

b. Transfect into PC3 cell and establish GFP-MKLP1 expressing cell lines. We expect most cells will express cytoplasmic GFP-MKLP1 whereas a subpopulation will express GFP-MKLP1 that localizes to MBds (putative cancer stem cells). Months 2-6

c. Isolate subpopulation of PC3 cells containing MBds from those that lack MBds using flow cytometry methods that detect object (MBd) size and intensity as we previously did for HeLa with Amnis Imagestream Technology. Months 4-8

d. Quantify % cells with MBd # and #MBds/cell. Months 6-10

e. Test MBd+ versus MBd- cells for transformation properties in vitro (count # colonies formed after plating, and colony # grown in soft agar). Test ability of cells to form xenograph tumors in mice. Initial experiment use 5 mice and inject 10^6 cells as done for PC3 populations. Repeat and adjust cell # or time to optimize tumor formation of tumors in MBd+ cells and compare with MBd- cells. Use statistical analysis to determine significance. Months 8-24

f. If dramatic differences are observed between MBd+ and MBd- cells, we will test subpopulations of MBd+ cells with *different MBd #s/cell* (potentially different stem cell lineages) in cell transformation and tumor induction assays. Months 24-36

Task 3 (Aim 3): **Test whether MBd disruption alters tumor potential of prostate cancer cells.**

a. Disrupt MBd inheritance in PC3 cell lines by randomizing the asymmetric distribution of MBds using microtubule depolymerizing agents or RNAi-mediated depletion of MBd proteins. Months 12-24

b. For microtubule depolymerization studies, treat cells with nocodazole concentrations that do not affect spindle organization but free MBds in cell.

c. Determine if MBds are segregated randomly as expected using long-term time-lapse imaging methods (24-36 hours).

d. Test cells that lose or gain MBds for changes in cell transformation and tumor induction assays.

e. For MBd disruption experiments in human and mouse tumors, prepare cells from tumors, transfect with siRNAs, confirm protein depletion. Months 20-24

f. Test cells MBd+ and MBd- cells for their ability to form tumors in mice and for transformation properties in vitro as above. Months 24-32

IMPACT

Our work changes the way we perceive the fundamental process of human cell division. It shows that multiple asymmetries during the final stage of cell division produce cells that are not born equal, but rather have distinct characteristics, consistent with older and younger cells. This shows that epigenetic inheritance of two complex organelles during cell division (midbody derivatives, MBds, and the older centrosome) may directly influence the properties of daughter cells resulting from cell division in ways that were previously unappreciated. In this application, we address the significance of the different daughter cells in putative prostate cancer stem cells.

This proposal is focused exclusively on prostate cancer because we have acquired experience in prostate cancer over the last several years (see publications in Doxsey Biosketch: 1998, 1999, 2000, 2001, 2003a, 2003b). The work in this proposal has important relevance for prostate cancer:

1. Prostate cancer etiology. The proposed studies have the potential to uncover novel and previously unappreciated mechanisms underlying prostate cancer etiology. These mechanisms, related to MBd inheritance, are associated with the fundamental process of cell division, which is at the core of cancer development and progression. To this end, we will test the role of MBds in prostate cancer stem cell viability, 'immortality' and tumor potential.

2. Prostate cancer stem cell markers. The proposed studies could identify prostate cancer-specific stem cells that contribute to human prostate tumorigenesis and progression. Using MBds as prostate cancer stem cell markers would be novel in that this 'marker' would actually represent an organelle not simply a molecule associated with stem cells. Currently, there are no good markers for prostate cancer stem cells. The MB is likely comprised of hundreds of proteins, which offers the potential to target any of these proteins for marker development. In separate studies, we are using proteomic analysis of the prostate cancer cell MBds to determine their molecular composition.

3. Prostate cancer prognosis/diagnosis. If the initial stages of this work are successful, future studies will focus on testing whether our current MBd proteins (or others) have prognostic and/or diagnostic potential. It is possible that the presence of MBd-containing prostate cancer stem cells indicates a poor prognosis.

4. ***Prostate cancer therapeutics.*** Another exciting future goal is to test the potential of targeting MBds for human prostate cancer prevention or elimination.

This proposal is innovative in many ways.

1) Our observation that two prostate cancer cells resulting from a cell division event are not born equal (that one inherits the midbody) is a novel concept. It deviates fundamentally from previous models of cell division and changes the way we perceive the process and the outcome of cell division. This new observation is the foundation for these studies.

2) We plan to examine the novel possibility that an entire cellular organelle comprised of hundreds of proteins, (the midbody derivative, MBd), is present selectively, if not specifically, in putative prostate cancer stem cells.

3) We will test the novel idea that components of the MBd will serve as putative prostate cancer stem cell markers.

4) The idea that MBds in putative prostate cancer stem cells have the potential to contribute to the etiology of prostate cancer development and progression is a new and exciting concept. This will be tested in this proposal.

5) The potential use of MBds for therapeutic targeting prostate cancer stem cells in future studies is a new concept in prostate cancer treatment.

PROPOSAL BODY

BACKGROUND AND PROGRESS.

Prostate carcinoma is the most prevalent gender-specific cancer in the USA[1]. The lifetime risk of developing invasive prostate carcinoma in the United States stands at ~20%[2-5], while that of octogenarians, based on histopathologic examination of the prostate at autopsy, approaches 80%[6]. Despite such an alarmingly high incidence, the lifetime risk of dying from prostate carcinoma is much lower, currently estimated to be around 3.6% (1/28, Surveillance Epidemiology, & End Results Website at NCI, 2001). The trend toward higher incidence and lower mortality will increase in the next few decades due to the combination of two factors: 1) the aging of the Baby Boom generation, which will result in an increase in the population at risk for this age-dependent cancer, and 2) the clinical implementation of ever more sensitive assays for prostate specific antigen (PSA), which are able to detect increasingly smaller cancer burdens long before the development of clinical symptoms. Yet, there remains an urgent need for better prognostic tests and more efficacious, less debilitating treatments for the disease. These will come from a better understanding of disease etiology and better prognostic indicators of the disease.

Our previous D.O.D.-funded work on prostate cancer over the last decade has focused on defects during mitosis that induce aneuploidy/genomic instability in prostate carcinoma and pre-invasive lesions (see Doxsey Biosketch: Pihan et al, 1998, 1999, 2001, 2003a, b). In continuing studies on this topic, we unexpectedly found that the centrosome protein centriolin functioned in an under explored area of mitosis called abscission, the final event in cell division where cell is cleaved apart to create the two new daughter cells[7]. Cleavage failure generates polyploid cells that ultimately missegregate chromosomes and become aneuploid[8, 9]. In our continuing investigations on abscission, we unexpectedly discovered that prostate cancer cells (PC3, DU-145) and others (HeLa, U2OS) underwent a series of asymmetric events during cytokinesis that generated daughter cells that were not identical as previously believed[7]. Instead, only one of the two daughter cells inherited the midbody (MB)--a singular organelle comprised of hundreds of proteins [10] that is assembled between two dividing daughter cells during cell division[7]. We call the asymmetrically inherited post-mitotic MB, the midbody derivative (***MBd***). Events leading to asymmetric inheritance of MBs in one daughter cell are shown in Fig. 1.

During human cell division, two replicated sets of chromosomes (DNA, blue, Fig. 1) must be segregated to opposite ends of the cell. The cell must then be severed to create two new progeny. ***In our recent studies, we unexpectedly uncovered five novel asymmetric events that cooperated to generate daughter cells that are not equal[7].*** 1) In the first step, membrane vesicles (green dots, Fig. 1) move asymmetrically to *one side* of the compact midbody (MB, black ring, Fig. 1A, B). 2) The vesicles fuse coordinately with the cell membrane and with one another to sever the cell in two (Fig. 1B, C). This defines a new role for vesicle fusion in human cell division. 3) The midbody moves to the cell opposite the severing site (Fig. 1C). 4) The cell with this midbody derivative (MBd) also contains the original centriole (Fig. 1C, red asterisk), a structure that replicates once every cell division cycle and contributes to mitotic spindle formation and chromosome segregation[9, 11, 12]. 5) Remarkably, in subsequent cell divisions MBds *accumulate* in this same cell (Fig. 1D). Thus, one cell receives MBds and the original centriole, whereas the other receives no MBds and a centriole copy. ***To our knowledge, there is no other example in cell biology where a singular organelle is asymmetrically inherited by one of the two daughter cells.*** The MBd localizes to the cytoplasm of the cell, lying just under the plasma membrane and actin

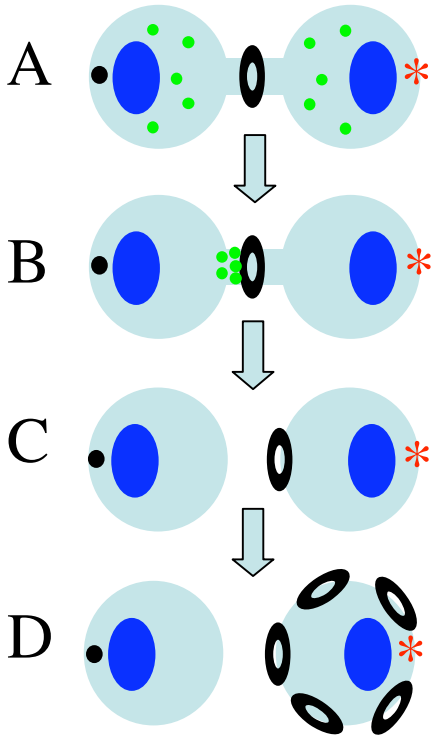


Fig. 1. Asymmetric cell division.

cortex. It is usually found in the vicinity of centrosome suggesting that it may be tethered to this structure by microtubule fibers emanating from the centrosome. With time the MBd loses structures (microtubules) and molecular components of the original MB. It is unlikely that these asymmetric events, which culminate in asymmetric inheritance of MBs, are random. It is also unlikely that asymmetric inheritance of MBds has no functional consequence. The focus of this proposal is to understand the functional consequences of MBd inheritance in prostate cancer cells.

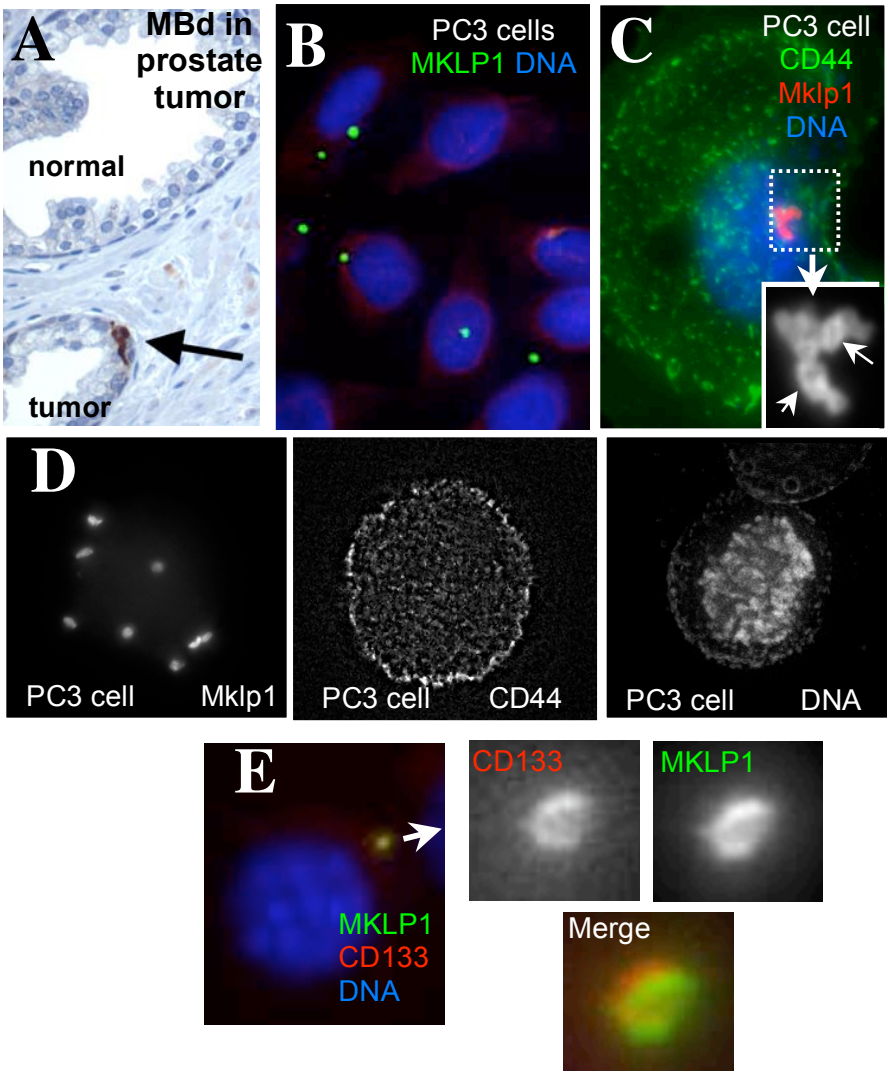


Fig. 2. See text for details.

MBds may be in putative prostate CSCs. To understand more about the inheritance and functional significance of MBd inheritance, we examined MBds in human and mouse prostate tumors and prostate cell lines (Fig. 2, next page). In paraffin and frozen sections of prostate tumors using immunofluorescence and immunohistochemical methods, we identified rare tumor cells that stained for MBd proteins (Fig. 2A, MKLP1). These cells were often adjacent to the basal layer in early lesions, the position of putative prostate CSCs (Fig. 2A). We have not yet co-stained for prostate stem cell markers in prostate tumors. Most other cells in the gland did *not* stain for MBds including non-basal cells that were positive for phospho-histone H3, a marker for mitotic (dividing) cells. We also found multiple MBds in many cells of the prostate cancer cell line PC3 (Fig. 2B-E, MKLP1); other prostate cell lines have not been examined rigorously. To test whether MBd-containing PC3 cells were putative prostate CSCs, we stained for presumed prostate CSC markers. We found that the MBd-containing cells were positive for CD44 (two examples: Fig. 2C, enlargement at arrow shows MBds, red, and Fig. 2D, all panels from the same cell) and CD133 (Fig. 2E)[13, 14]. Only a subset of the CD44+ or CD133+ cells had MBds suggesting that MBd-containing cells have higher stem cell potential than the CD44+ or CD133+ cells. CD44+ cells had a very high number of MBds compared with CD44- cells (Fig. 2D). CD133 localized to MBds in PC3 cells (Fig. 2E, enlargements right) suggesting a possible function for the protein at this site. MBds did *not* localize to normal primary prostate epithelial cells. This work suggests that MBd-containing cells may be prostate CSCs. This idea is consistent with the emerging concept that prostate cancer develops from malignant transformation of stem cells, those cells that divide to produce a stem cell (self-renewal) and a cell that ultimately differentiates[15-17]. Other prostate CSC markers have not yet been tested.

The field of prostate stem cells in cancer is still in a nascent stage[15, 17]. The cell of origin of prostate cancer is still unknown. As stated in a recent review on this topic: "The identification of [prostate CSCs] depends on understanding prostate cell differentiation…as well as adult prostate epithelium renewal"[15]. To this end, we examined several normal tissues from human and mouse for the presence of MBds. Since neither markers nor

architectural clues definitively identify the prostate stem cell, we first examined putative stem cells in other tissues (Figs. 3-5, Table 1, next page). We found that MBds were confined to sites of stem cells in the testes (basal layer, Oct4+/MELK+, Fig. 3)[18], hair follicle (a small subset, 1/1000, of K15+ cells, Fig. 4)[19], gut (+4/5 position from bottom of crypt, Fig. 5)[20] and brain (subventricular zone, radial glia/astrocytes, GFAP+, nestin-)[21]. Moreover, cultured human embryonic stem cells (H9) had multiple MBds (Fig. 6), which were dramatically reduced when cells were induced to differentiate into neurons. Importantly, MBds were also found in cancer cells of different origins and their prevalence was rather variable (Table 1). We have not tested whether this variability relates to tumor potential of these different cell lines. MBds were *not* observed in dividing *non-stem* cells such as hepatocytes in regenerating mouse liver, T cells activated ex vivo and transit amplifying cells in all organs discussed above (Table 1). Similarly, MBds were *not* found in normal diploid cell lines (mouse embryo fibroblasts [MEFs], RPE, hMEC). Taken together, these results suggest that MBds are in normal stem cells and CSCs in vivo and in vitro, including those of the prostate.

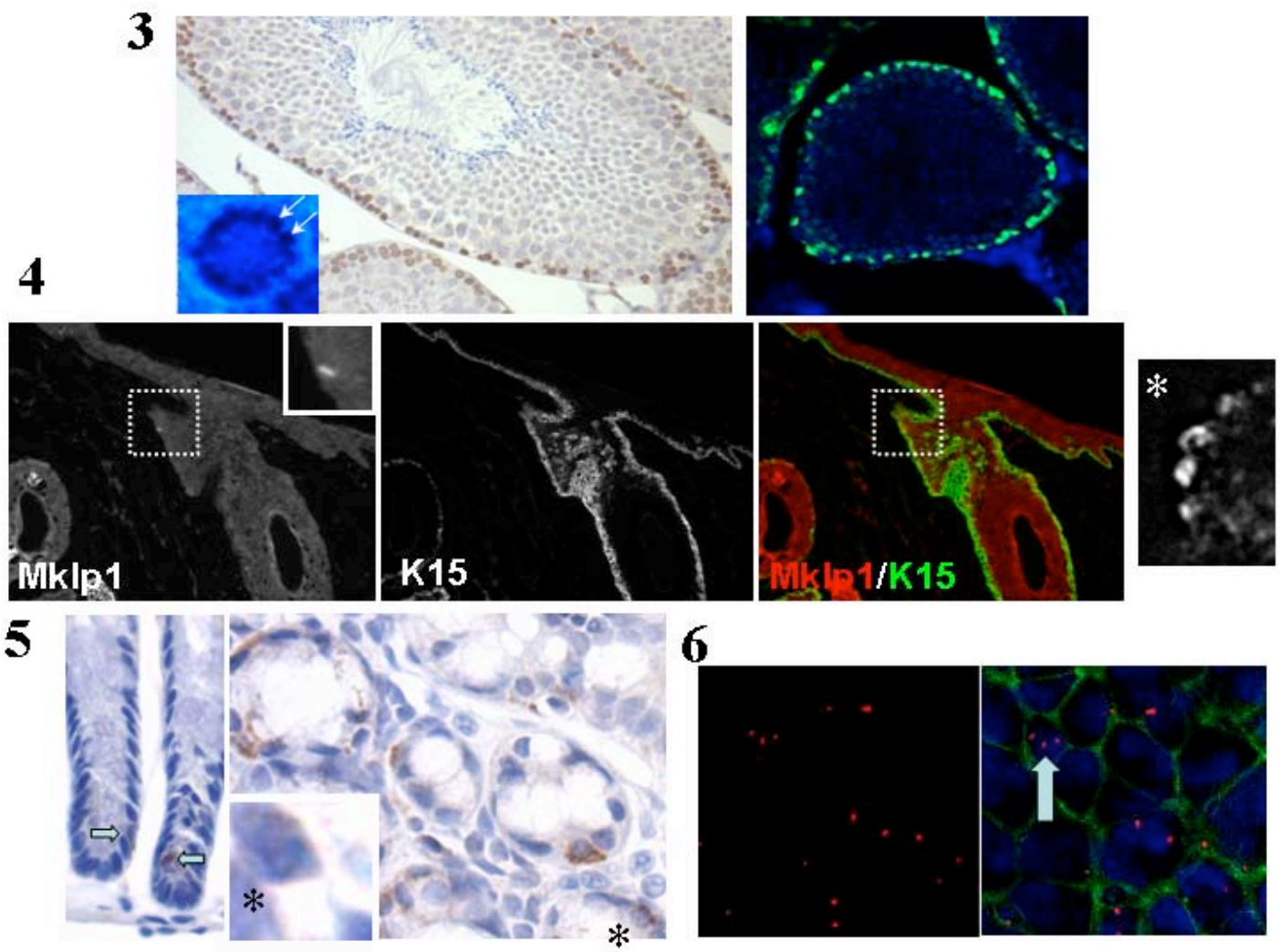


Figure legend. Fig. 3. Mouse testes histological section stained for MB^{d}s (left, brown color) and counterstained with hematoxylin (blue). Staining is largely confined to the basal cell layer (stem & pr ogenitor cells). Enlargement (bottom left) shows that staining in this individual cell is punctate, as expected for the presence of MB^{d}s. At right is testes stained for a stem cell marker (Oct 4) showing a similar pattern to MB^{d}s stain. **Fig. 4.** A section through a hair follicle (left, box enlarged upper right) shows that a small subset of cells that are positive for the epithelial marker K15 (middle, marks the stem cell compartment) stain for MKLP1 (MB^{d}s). Right, color merge. Far right image (*) shows ring-like structures stained with MKLP1 in another skin section (immunofluorescence)**. Fig. 5.** Section of mouse colon showing cells in a plane consistent with the +4/5 position (stem cells), stain for MB^{d}s. Inset, right higher magnification of cell at * showing multiple MB^{d}s. **Fig. 6.** MB^{d}s (left) in H9 embryonic stem cells (right, merged with nuclei and actin). Arrow, cell with three MB^{d}s.

Table 1. MB derivatives are found in stem cells

Cell type	% cells w/MBds)
Human Embryonic SCs	
H9	51.7
H9 after differentiation	4.6
NCCIT (hESC tumor)	66.8
Non-stem cells	
RPE1 (human)	0.9
Mouse tracheal cells	0.2
Mouse embryo fibroblasts	0.7
Human cancer cell lines	
MDMB231	7.2
293T	29.1
HeLa	21.1
PC3	<15

Are these CSCs?
(n=4-6000 cells counted for each cell line)

Tissue type (Location)
Testes stem cell compartment (basal layer, MELK1+ cells)
Skin stem cell compartment* (bulge, K15+ cells)
Gut stem cell compartment (+4/5 cells in crypts)
Breast (rare cells near basal lamina)
Prostate (rare cells near basal lamina, CD133+)
Activated mouse T-cells (no MBds)
Regenerating mouse liver (no MBds)
Transit amplifying cells in above organs (no MBds)

Are MBds a universal stem cell marker?
Do MBds contribute to stem cell self-renewal?
**MBds decrease with age in human skin:*
(elderly<adult<fetal)

HYPOTHESIS.

Based on our preliminary data we hypothesize that MBds will serve as markers for prostate CSCs and could also have diagnostic/prognostic value for prostate cancer progression. The remarkable ability of putative prostate CSCs to accumulate MBds through a complex series of asymmetric events strongly suggests that these MBds could contribute directly to the etiology of prostate cancer. In fact, a protein associated with stem cells of high potency, CD133/prominen, is found at the midbody (MBd)[22]. Perhaps other stem cell markers are located to MBds as well. This proposal is focused on prostate CSCs as our laboratory has long-standing experience in prostate cancer research and our research group has all the required expertise to complete this project. See biosketches of Drs. Doxsey, Houghton, Lyle, Altieri) and also letters of support from Drs. Houghton, Lyle, Altieri.

SPECIFIC AIMS.

The long-term goal of this project is to identify putative MBd-containing prostate CSCs and target them for chemotherapeutic intervention in prostate cancer. Toward this goal we will determine if MBds are present in putative prostate cancer cells from human tumors and cell lines (Aim 1), isolate MBd-containing cells and directly test them for tumorigenic potential in mice (Aim 2) and determine if loss of MBds from these cells diminishes their tumorigenic potential (Aim 3). The Specific Aims are:

Aim 1. Determine if MBds are present in prostate CSCs.

a. Determine if MBds are in prostate cancer cells expressing *putative* stem cell markers using cell lines, cells dissociated from human tumors and tissue sections.
b. Determine if MBds are in prostate cancer side populations in tumors and cell lines.
c. Determine if MBds are in BrdU (label) retaining prostate cancer cells in tumors and cell lines.

Aim 2. Isolate and test MBd-containing prostate cancer cells for CSC properties.

.

a. Construct PC3 cell lines expressing GFP-tagged MBd protein (e.g. MKLP1) and isolate these cells using flow cytometry based on object (MBd) size and intensity.
b. Test MBd+ and MBd- cells for their ability to form tumors in mice and for tumor properties in vitro.
c. Test other prostate cancer cell lines with different tumor potential (PC3>DU-145>LNCaP).

Aim 3. Test whether MBd disruption alters tumor potential of prostate cancer cells.

a. Isolate MBD+ PC3 cells (Aim 2) and disrupt asymmetric MBd inheritance using microtubule depolymerizing agents or eliminate MBds by RNAi-mediated depletion of MBd proteins. Test MBd+ and MBd- cells for tumorigenicity in Aim 2.
b. Disrupt MBds in cells isolated from excised human and mouse (PTEN-/-) prostate tumors by siRNA. Test for tumorigenicity in Aim 2.

The studies proposed in this application are part of a larger effort to examine mechanisms and roles of MBds in cellular functions. Other projects will test the role of MBd inheritance in normal human stem cell function, in cellular 'aging' and in aging of the organism. These projects are independently funded by NIH and NCI, and we anticipate funding from the Keck Foundation, Ellison Medical Foundation and UMass President's Science/Technology Initiative. These other studies will involve genome-wide RNA interference screens and MBd proteomes that will identify MBd genes to be exploited for targeting prostate cancer cells in this study. The prostate CSC work described here is not currently funded, nor is it a primary focus of pending proposals.

RESEARCH STRATEGY

If successful, the studies outlined in this proposal will demonstrate that MBds are present in prostate CSCs and will determine if MBds contribute to prostate CSC self renewal. This work should proceed rapidly and efficiently, as our research team has expertise in all aspects of this project (prostate cancer techniques, cells, IRB-approved protocols for human and mouse experiments, imaging, etc.).

Specific Aim 1. Determine if MBds are present in prostate CSCs.

Rationale. It has been suggested that prostate tumors contain sub-populations of cells with stem cell qualities called "cancer stem cells (CSCs)"[15, 16]. This aim is designed to test if MBds are present in putative prostate CSCs. Although none of current markers or assays definitively identifies prostate CSCs, together they provide a working model to test whether MBds are found in these cells. The goal of this proposal is *not* to identify a prostate cancer cell with the highest stem cell potency, but rather to simply determine if MBds are present in cells that have stem cell properties. In fact, our preliminary work (above) suggests that MBds are present in prostate cancer stem-like cells, as they are found in PC3 cells, they are in a subset of CD133+ PC3 cells, they are in a subset of CD44+ PC3 cells and they contain CD133 as an integral component [22] (Fig. 2). CD44 has been used to identify and isolate prostate CSCs from solid tumors and these cells are able to re-establish cancer cell populations in vitro and to form tumors when injected into mice; the non-stem cell fractions differentiate and die in vitro and do not form tumors in mice[14]**.** This shows that putative prostate CSCs can recapitulate properties of in vivo tumors, where a presumably rare population of stem cells divides asymmetrically to generate daughter cells with various levels of differentiation. We hypothesize that sub-populations of cells within prostate tumors and prostate tumor cell lines retain self-renewal properties of normal tissue stem cells. We propose that these cells divide asymmetrically and accumulate MBds. To identify and isolate putative CSCs we will use three strategies: stem cell markers, 'side populations' (SP) and BrdU-retention; combinations of these will also be used (e.g. isolation of side population followed by stem cell marker-

mediated isolation). Through this analysis, we hope to establish that MBds are consistently found in putative prostate CSCs.

Aim 1.a. Determine if MBds are in prostate stem cancer cells using putative stem cell markers. In this subaim, we will use a) cultured human cancer cell lines, b) cells dissociated from human prostate cancers and c) tissue sections containing prostate tumors. **a)** Prostate cancer cell lines and normal telomerase-immortalized diploid prostate epithelial cells (PrECs, control cell line)[23, 24] will be directly stained with putative prostate CSC markers using immunofluorescence methods employed in our previous work[25, 26]. Antibody markers will initially include MKLP1 and Cep55 for MBds; other markers will be used as they are identified in parallel studies (MBd proteome, shRNA screens, above). Putative prostate CSC surface markers will initially include CD133 and Cd44, as we have co-localized these molecules to MBds (above). Other positive (and negative) markers will be used as needed (see[15]). MBds will be quantified in two ways (see Table 1). The percent of cells with MBds will be accurately determined by counting cells stained with MKLP1 antibody. This data will be confirmed with Cep55 antibodies. The data from both antibodies should be the same as they both localize specifically to MBds. This will be further confirmed by demonstrating that every fluorescent ring/dot correlates with a phase-dense structure, as we have shown for MBds in our previous work[25, 27]. Similar analyses will be done on control cells (PrECs). Quantification of CD133 and CD44 staining is straightforward[13, 14]. For co-localization studies (MBd/CSC marker) we will use either monoclonal mouse or polyclonal rabbit MKLP1 antibodies, as they can be used in combination with CD133 or CD44 as well as other prostate CSC markers[13]. Co-localization studies will be done to determine what percent of CD133 or CD44 cells are MBd+ and, in turn, what percentage of MBd+ cells are positive for CD133 or CD44. In this way, we will obtain a comprehensive analysis of co-localization of these markers. Using a similar experimental design, we will determine the *number of MBds/cell* in these populations. This will be important in future studies, where we will use other prostate cancer cell lines to determine if MBd number or the percentage of MBd+ cells in the total population correlates with tumor potential: LnCAP (low), DU-145 (medium), PC3 (high)[14]. **b)** For fresh human prostate tumors, we will digest tissue to single cells and remove contaminating fibroblast, endothelial and neural cells with magnetic bead-bound antibodies. Cells will be stained for CSC markers then isolated by FACS. They will be cytospun onto coverslips, detergent-permeabilized to provide access to intracellular MBds and processed for MBd staining to determine what percent of the CSC marker+ cells contain MBds. In addition, marker-negative fractions will be stained for MBds, as this should serve as a negative control (if MBds are always found with the CSC marker). **c)** Prostate tumor tissue sections will be processed for co-staining of putative prostate CSC markers and MBds or by labeling of successive serial sections from the same tumor with individual antibodies, as in our previous studies[28, 29]. We will use both archived paraffin and frozen tumor tissue representing different histological subtypes of human prostate cancer. Frozen sections give better resolution of structure, and double-labeling is more straightforward than on paraffin sections. For the experiments described above and throughout this proposal, statistical analysis will be done as in our previous studies[15, 30, 31].

Expected outcomes, ***potential problems and solutions.*** We have shown that MBds are found in a subset of CD133 cells and of CD44 cells but this has not been accurately quantified. Based on our preliminary data, we expect that MBds will be in about half of CD133-labeled PC3 cells; we do not have sufficient data to estimate the proportion of CD44/MBd co-staining. In either case, our expectations are consistent with MBds being present in PC3 cells with high stem cell potency. One potential problem in the tumor dissociation study is that contaminating stem cells from normal tissue may be present. So, in addition to this analysis, we will also generate xenograph tumors from PC3 cells in the skin of mice. In this way, we can unequivocally identify the CSCs using in the tumor setting. For example, if necessary we can use antibodies that differentially target human versus mouse HLA/MHC antigens. Another potential issue is that a large quantity of tissue may be required to recover adequate numbers of dissociated cells from fresh prostate tumors. However, this will be feasible as our research team includes the Director of the Tumor Tissue Bank (Dr. Steve Lyle, see letter of support) who receives 2-5 prostate tumors/week and has protocols to retrieve large amounts of tumor tissue.

Finally, during the course of this study we will test other putative prostate CSC markers for localization to the MBd, as this will further implicate MBds in prostate CSC function.

Aim 1b. Determine if MBds are in prostate cancer side populations. Prostate cancer cell lines (and dissociated prostate tumor cells) can recapitulate properties of in vivo tumors[14]. These rare populations can be enriched since they express the multi-drug resistance transporter on their cell surface. This allows active efflux of the Hoechst 33342 DNA-binding dye causing a shift away from the normal labeled population detected by FACS (side population, SP)[14, 32-34]**.** Inhibition of the transporter by the drug verapamil shifts cells back into the main population, confirming the presence of the transporter. The transporter does not likely confer stem cell self-renewal properties to a cell, but provides a useful mechanism to enrich for putative stem cells within certain cell populations.

We will take advantage of this side-population (SP) phenomenon to enrich for putative prostate CSCs. Side populations have already been isolated from PC3, LnCaP and Du-145 cell lines and have been shown to be tumorigenic[35]. In fact, the side populations of these cells exhibit different tumor potential (PC3>DU-145>LNCaP). We will use these cells and this system to isolate side populations containing putative prostate CSCs. The CSC phenotype will be verified by standard in vitro clonogenic growth and anchorage-independent assays, and tumor formation assays in mice (details in Aim 2b)[14]. We will test for the presence of MBd markers in SPs and compare these to non-SP cells from the same experiment, as well as non-fractionated whole cell cultures and the main population (MP). We will use the same rigorous quantitative and statistical methods described in Aim 1a to identify differences between SP and non-SP populations. We will also use this strategy to test whether the percent of SP cells with MBds or the number of MBds/cell correlates with tumorigenicity in the three different prostate cancer cell lines.

Expected outcomes, ***potential problems and solutions.*** Because SP cells are enriched for CSCs but are not specific for CSCs, we expect MBs will localize to a sub-population of the SP cells. *This will be tested more directly by isolating SP followed by a second round of enrichment with stem cell markers (described in Aim 1a).* Because cell lines can become modified when adapted to culture, we will also perform SP analyses on freshly-isolated cells dissociated from human prostate tumors. To accomplish this, we will prepare cells from human prostate tumors as described in Aim 1a and analyze this SP for MBds. Again, one problem with this approach is that normal SCs may contaminate the tumor. However, if normal prostate gland tissue is as a control for normal SCs, we can get a better idea of the proportion of CSCs in this population. This experiment is worth the effort as it involves the study of primary human tissue.

Aim 1c. Determine if MBds are in prostate cancer cells that retain BrdU for long periods of time (label-retaining cells). A powerful method for identifying epithelial stem cells takes advantage of the slow-cycling nature of such cells. In this procedure, a tissue is long-term labeled with 3H-thymidine or BrdU so that all cells, including the stem cells, are labeled. This is followed by a "chase" period during which the label is diluted out from all the rapidly dividing (transit amplifying) cells but is retained by the slow-cycling cells (including stem cells), which can thus be identified as the "label-retaining cells". This method has recently been used to locate prostate stem cells in mice[36].

We will take advantage of this method to label putative prostate CSCs in PC3 cells as recently described[14] and assay them by standard in vitro clonogenic growth assays and tumor formation assays[14] (see Aim 2b). Future studies will include identification and co-staining of label retaining cells in prostate tumors in PTEN-/- mice with MBd markers using established protocols for labeling putative prostate CSCs in vitro and in vivo[14, 37]. My colleague, Dr. Roger Davis (UMass) has PTEN null mice and has offered them to us for these experiments. To provide a more tumor-like environment for this study, we will also label cultured cells with BrdU and produce xenograph cancers, re-isolate the cells and identify label-retaining cells and their relationship with MBds.

Specific Aim 2. Isolate and test MBd-containing prostate cancer cells for CSC properties. In this aim, we will directly test the tumorigenic potential of MBd-containing prostate cancer cells. Our preliminary data demonstrate that we can 1) produce stable HeLa cell lines expressing GFP-MKLP1, 2) identify cells with fluorescent GFP-MKLP1- labeled MBds and 3) distinguish them from those cells that *lack MBds,* based on their intense focused fluorescent signal(s) using FACS analysis and Amnis ImageStream software (Dr. Richard Konz, Director, UMass FACS Core facility). However, we have not sorted or characterized any cell line containing GFP-tagged MBds. In this aim, we will construct prostate cancer cell lines (PC3) expressing GFP-tagged MBd markers then isolate cells with GFP-tagged MBds and compare them to GFP cells without MBds (cytoplasmic fluorescence). This method should be feasible and straightforward given our success with construction of GFP-MKLP1-expressing Hela cells and detection of cells with GFP-tagged MBds. The prostate cancer cell lines will not only be useful in tumorigenic assays but will have future utility in the isolation of MBds for proteomic analysis (future goal) and for comparisons of MBds in cells with different tumor potential (Pc3>DU-145>LNCaP).

2a. Construct and select PC3 cell lines expressing GFP-MKLP-1 or GFP-Cep55, two markers for MBds. Initially, we will construct PC3 cell lines expressing GFP-MKLP1 under control of the CMV promotor as done for HeLa cells. Cell lines will be generated by selection for the GFP plasmid under G418. In all studies, we will use at least two different isolates if each cell line to ensure reproducibility of the data. The MBd-containing fractions will be identified by FACS (as with HeLas) and cells will be selected based on the presence of fluorescent MBds (bright objects of defined size). The presence of MBds in selected cells will be confirmed by cytospinning the appropriate cell fractions onto coverslips and imaging GFP-tagged MBds by high resolution spinning disc confocal microscopy in our laboratory[7, 26, 27]. MBd-containing cells will be identified by the presence of fluorescent rings of 3-4 micrometers, a hallmark of these structures[7]. We will also confirm that cells negative for bright object intensity by FACS, lack GFP-tagged MBds by immunofluorescence staining. Finally, the presence of MBds will be tested by immunoblotting for additional MBd components (e.g. Cep55, MKLP1, survivin, others). ***Anticipated outcome***. Based on our HeLa cell GFP-MKLP1 cell lines we should be able to successfully separate MBd-containing PC3 cells from the general population.

Aim 2b. Test MBd+ and MBd- cells for their ability to form tumors in mice and for tumor properties in vitro. Once GFP-MBd+ and GFP-MBd- PC3 cell populations are obtained we will test for differences in their ability to form tumors in mice. PC3 cells are known to form tumors when injected subcutaneously in NOD-SCID mice and they exhibit tumor properties in vitro. For in vitro studies inthis aim and others, we will analyze cells for the number and size of colonies following growth in soft agarose as done in our previous studies[29, 30]. Briefly, 10^5cells will be plated in triplicate in six-well plates in 0.35% agarose over a cushion of 0.7% agarose and assessed for colony appearance and size 3 and 7 days after plating using an inverted microscope by photographing no fewer than 10 fields. We will also use a "self-renewal" assay, the clonogenic assay[14], which will involve plating cells (in triplicate) at clonal density (100 cells/18mm^2 coverslip) and assaying for the percent of cells that initiate a clone as well as the size of clones over time for up to two weeks. This will provide a measure of the cloning efficiency, the ability of one cell to give rise to a colony. ***Xenograph tumor assays*** for this aim and others will be performed as in previous studies by our collaborator Dr. Dario Altieri (Director, UMass Cancer Center/Cancer Biology Department, see letter of support)[30]. Briefly, NOD-SCID mice will be injected in the flanks with PC3 cells (~2.5 x 10^6 cells, 4-6 animals per group) and allowed to form tumors (~18 days, tumor volume ~1-2 x 10^3 mm^3). Tumors will be measured daily with a caliper, and tumor volume will be calculated according to the formula ½ (length [mm] x width [mm])^2. Initial studies will be performed using PC3 cells from the parent population to establish the timing of tumor growth and will be used as a gauge and control for both GFP-MBd+ and GFP-MBd- PC3 cells.

Anticipated outcome. We predict that MBd-containing cells will exhibit CSC properties similar to putative CSCs isolated by stem cell markers and FACS[15, 16] or by isolating side populations[14]. In fact, we predict that they will have higher CSC potential, as they represent a subset of cells labeled with putative CSC markers.

We predict the MBd-negative cells would have a diminished ability to proliferate and grow in soft agar. In fact, these cells may lose the ability to grow even under normal culture conditions and may differentiate and die in culture. For tumorigenic assays, we anticipate that the MBd+ cells will have properties of prostate CSCs and will grow tumors similar to or more aggressive than the parent PC3 cell line. The MBd-negative cells would be expected to grow poorly or not grow at all. ***Potential problems and solutions.*** Based on our strong data showing that MBds localize to stem cells and CSC fractions that have been shown to be tumorigenic, we predict that MBd+ containing cells will have CSC properties. If this is not the case, it is possible that the GFP construct interferes with this activity. To address this, we will make constructs with the GFP on the C-terminus of MKLP1 (the initial line will be an N-terminal GFP fusion). It is also possible that MBds co-segregate nearly perfectly with cells isolated by one of the other CSC procedures (e.g. one of the putative prostate CSC markers[15]). If this were the case, the protein would serve as a surrogate marker for MBd-containing cells. We hypothesize that the important parameter form MBd involvement in CSC biology may be MBd number/cell rather than the presence or absence of MBds. It is possible that *MBd number* is related to CSC properties. We will be mindful of this idea during the course of this study.

Aim 2c. Tumor growth of other prostate cell lines expressing GFP-MKLP1. As mentioned earlier, the tumor potential of side populations isolated from three different prostate cancer cell lines appears to be different, with PC3 being most aggressive, DU-145 being intermediate and LNCaP being least tumorigenic in xenograph tumor experiments[35]. If we find that MBd+ PC3 cells are more aggressive in xenograph tumor growth than the MBd- cells or the parent PC3 cell line as we expect, then we will use test DU-145 and LNCaP cell lines to determine if the MBd+ population behaves in similar way to the side population. In other words, we will test whether the tumor induction potential of MB+ cells mimics the tumor induction potential of side populations from all three cell lines (PC3>DU-145>LNCaP). This analysis will provide further evidence that the MBd+ cells are putative prostate cancer cells. ***Potential problems and solutions*** are as in Aim 2b.

Specific Aim 3. Test whether MBd disruption alters the tumor potential of prostate cancer cells. Thus far, we have shown that MBds are present in many different types of cancer cells in vitro and in vivo. However, we do not know the functional significance of this association. Is MB accumulation required for prostate CSC maintenance or is it merely a marker for these cells? In this aim, we will directly test the role of the MBd in prostate CSC tumorigenesis. MBds will be disrupted using several strategies and the effect on tumor properties in vitro and tumor induction in mice will be examined as in Aim 2b.

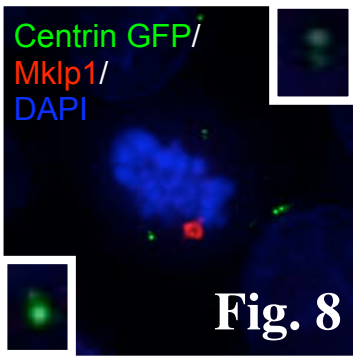


Fig. 8

Aim 3a. Test the effect of MBd disruption on xenograph tumor formation. For this study, we will isolate the GFP-MKLP-tagged MBd containing cells from the parent cell line, GFP-MKLP1 PC3 cells. (GFP-MKLP1 DU-145 and LNCaP cells will also be constructed and used if PC3 experiments are successful). MBds in these cells can be visualized in the following experiments using time lapse imaging as in our previous studies[25]. MBds will first be disrupted by microtubule depolymerization. Preliminary results show that MBds in HeLa cells (Fig. 8, red) are clustered around the older pole of the mitotic spindle (Fig. 8, brighter centrin dot, green, enlarged in inset at bottom left, DNA, blue). MBd clustering occurred in 92.2% of the cells (n=51). This demonstrates that MBds are asymmetrically organized *before* they initiate cytokinesis. Because the older centrosome organizes more microtubules, it is possible that these microtubules contribute to the asymmetric localization of the MBd during mitosis. In fact, we have used low concentrations of the microtubule depolymerizing agent nocodazole to disrupt microtubule dynamics while retaining spindle integrity and function, and we find that MBd localization is randomized in these spindles (55.5%, n=36, Ting Chen, unpublished observations). Based on this observation, we will treat cells with nocodazole to randomize MBd inheritance during mitosis. Continued randomization with successive divisions, will lead to a decreased number of MBds/cell and possibly loss of CSC characteristics. Randomization of MBd inheritance will be detected by time-lapse fluorescence imaging over several days in a heated chamber[25] (Wadzinski et al, J Cell Biol, in press) and by FACS analysis based

on GFP object size and intensity (see Aim 2). The tumor potential of these cells will be examined in vivo as described in Aim 2b using similar animal numbers for xenograph tumors and in vitro assays (proliferation, clonogenic analysis, focus formation, soft agar assay). Again, xenograph tumor studies will be done collaboratively with Dr. Altieri (see support letter).

In another strategy, we will eliminate MBds by RNAi-mediated depletion of MBd proteins. We will initially target the MBd protein Cep55, as this protein is required for midbody integrity[38]. We have a great deal of experience in siRNA and shRNA-mediated gene silencing[7, 25, 26]. Additional MBd proteins will be used as they be come available from our parallel studies funded by other institutions that are focused on identification of MBd proteins through determination of the cancer cell MBd proteome and shRNA library screens (above). Using this strategy, we are confident that this approach will succeed.

Anticipated outcome, potential problems and solutions. Our model predicts that disruption of MB function should decrease tumorigenicity through loss of CSCs. We will be mindful of the possibility that the GFP tag may affect protein and cell function. If we encounter difficulties with the GFP-MKLP PC3 cells, we will repeat these experiments in PC3 cells without the GFP tag. This strategy will be dictated by results in Aim 2 where these GFP cell lines will be tested. Nocodazole can affect all microtubules of the mitotic spindle although our results suggest that the MBd-associated microtubules are more sensitive to the drug, providing a window of opportunity for specific MBd randomization. The siRNA depletion studies have the potential to affect cell division and ploidy if the mitotic midbodies are targeted[38]. However, a recent study shows that a tissue-specific knockout of a well-established cytokinesis gene (Rac1), does not induce polyploidy, but rather specific stem cell depletion[42]. We expect that Cep55 depletion will produce a similar phenotype in vitro. If this is not the case, MBd-specific genes will be targeted as they are identified in shRNA and chemical screens performed in parallel in collaboration with Novaratis and with other funding. Nevertheless, the use of two complementary strategies to address the role of MBds in CSC biology optimizes our chances of success.

Aim 3b. Test the effect of MBd disruption in prostate cancer cells excised from human and mouse tumors. In this subaim, we will utilize human prostate tumors and mouse tumors generated in PTEN/- mice, which most accurately resemble human prostate cancer progression[39]. Tumors will be excised and cells derived from these tissues will be grown in culture. Cultures will be treated with Cep55 siRNA using methods similar to our previous studies[25, 26]. Cep55 protein levels will be examined at various times after siRNA transfection by immunoblotting and immunofluorescence to confirm protein depletion. Cells treated with Cep55 siRNAs and controls (scrambled, GFP) will be tested for their ability to proliferate, form colonies in soft agar, clones on coverslips and induce xenograph tumors in mice (see Aim 2). If we observe an effect on tumor induction, we will perform additional studies to identify the mechanism of Cep55 action (below).

Anticipated outcome. We predict that prostate CSCs will be eliminated following Cep55 depletion. This would occur through loss MBds in CSCs, thus preventing tumor growth (if prostate cancer is solely reliant on stem cells). Under these conditions, one would expect a concomitant loss of stem cell markers from these cells (e.g. CD133, CD44, CXCR4)[13] and an increase in differentiation markers[15]. Markers will be monitored by FACS and fluorescence imaging as described previously. One would also expect that MBd-depleted cells isolated by FACS on the basis of CSC markers would fail in tumor induction assays, since the stem cells would be lost. In contrast, control siRNA treated cells would form tumors in this experiment. ***Potential problems.*** We have chosen to use excised material for this experiment rather than dual knockout of PTEN and Cep55 in the prostate, as it avoids targeting of Cep55 in normal prostate cells. Specific prostate CSC targeting (without affecting normal stem cells) remains a hurdle to overcome.

Cell, Vol. 123, 75–87, October 7, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.cell.2005.07.027

Centriolin Anchoring of Exocyst and SNARE Complexes at the Midbody Is Required for Secretory-Vesicle-Mediated Abscission

Adam Gromley,[1,3] Charles Yeaman,[2] Jack Rosa,[1] Sambra Redick,[1] Chun-Ting Chen,[1] Stephanie Mirabelle,[1] Minakshi Guha,[1] James Sillibourne,[1] and Stephen J. Doxsey[1,*]
[1]Program in Molecular Medicine
University of Massachusetts Medical Center
Worcester, Massachusetts 01605
[2]Department of Anatomy and Cell Biology
University of Iowa
Iowa City, Iowa 52242

Summary

The terminal step in cytokinesis, called abscission, requires resolution of the membrane connection between two prospective daughter cells. Our previous studies demonstrated that the coiled-coil protein centriolin localized to the midbody during cytokinesis and was required for abscission. Here we show that centriolin interacts with proteins of vesicle-targeting exocyst complexes and vesicle-fusion SNARE complexes. These complexes require centriolin for localization to a unique midbody-ring structure, and disruption of either complex inhibits abscission. Exocyst disruption induces accumulation of v-SNARE-containing vesicles at the midbody ring. In control cells, these v-SNARE vesicles colocalize with a GFP-tagged secreted polypeptide. The vesicles move to the midbody ring asymmetrically from one prospective daughter cell; the GFP signal is rapidly lost, suggesting membrane fusion; and subsequently the cell cleaves at the site of vesicle delivery/fusion. We propose that centriolin anchors protein complexes required for vesicle targeting and fusion and integrates membrane-vesicle fusion with abscission.

Introduction

Cytokinesis is a fundamental process that results in division of a single cell with replicated DNA into two daughters with identical genomic composition (see (Glotzer, 2001, 2005; Guertin et al., 2002). Early events in animal cell cytokinesis include assembly and contraction of the actomyosin ring to form the cleavage furrow. Continued furrowing results in constriction of the plasma membrane to form a narrow cytoplasmic bridge between the two nascent daughter cells. Within this intercellular bridge are bundled microtubules and a multitude of proteins that together form the midbody. In a poorly understood final step called abscission, the cell cleaves at the intercellular bridge to form two daughter cells.

Membrane trafficking is required for late stages of cytokinesis (Albertson et al., 2005; Finger and White, 2002; Jurgens, 2005; Papoulas et al., 2004; Strickland and Burgess, 2004). In *C. elegans* embryos, inhibition of Golgi secretion by brefeldin A (BFA) resulted in late-stage cytokinesis defects (Skop et al., 2001). More recent studies in mammalian cells using dominant-negative approaches showed that the membrane-fusion-inducing SNARE components, syntaxin-2 and endobrevin/VAMP8, are required for a final step in cell cleavage (Low et al., 2003). Endocytic traffic also plays a role in cytokinesis. Recycling endosomes and associated components localize to the midbody and are required for cell cleavage (Monzo et al., 2005; Wilson et al., 2005; Thompson et al., 2002). However, little is known about the spatial and temporal control of dynamic membrane compartments and molecules during abscission or how these activities are coordinated to achieve cell cleavage.

The role of membrane-vesicle-tethering exocyst complexes in animal cell abscission is poorly understood. The exocyst is a multiprotein complex that targets secretory vesicles to distinct sites on the plasma membrane. In the budding yeast *S. cerevisiae*, exocyst components localize to the mother-bud neck, the site of cytokinesis (Finger et al., 1998; Mondesert et al., 1997). Exocyst disruption results in accumulation of vesicles at this site (Salminen and Novick, 1989) and impairs actomyosin-ring contraction and cell cleavage (Dobbelaere and Barral, 2004; Verplank and Li, 2005). In the fission yeast *S. pombe*, exocyst components localize to the actomyosin ring (Wang et al., 2002). Mutants for the exocyst component Sec8 accumulate 100 nm "presumptive" secretory vesicles near the division septum and cannot complete extracellular separation of the two daughter cells. A screen for cytokinesis mutants in *Drosophila melanogaster* identified the exocyst component sec5 (Echard et al., 2004), and proteomic analysis of the midbody in mammalian cells showed that the exocyst protein sec3 is at the midbody (Skop et al., 2004). Mammalian exocyst components are involved in secretion in polarized epithelial cells (Yeaman et al., 2004) and localize to the midbody (Skop et al., 2004; Wilson et al., 2005), but the function of the exocyst during cytokinesis is unclear.

Components of membrane-vesicle-tethering and -fusion complexes have been identified in some organisms and linked to cytokinesis, but the pathway that integrates these complexes with vesicle trafficking during cell cleavage is unknown. Little is known about how SNAREs and the exocyst are anchored at the midbody or how they modulate membrane-vesicle organization and fusion to coordinate abscission. Moreover, the origin and dynamics of membrane compartments involved in abscission have not been investigated. In this manuscript, we describe a multistep pathway for abscission that requires a scaffold protein to anchor

*Correspondence: stephen.doxsey@umassmed.edu
[3]Present address: Department of Genetics and Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, Tennessee, 38105.

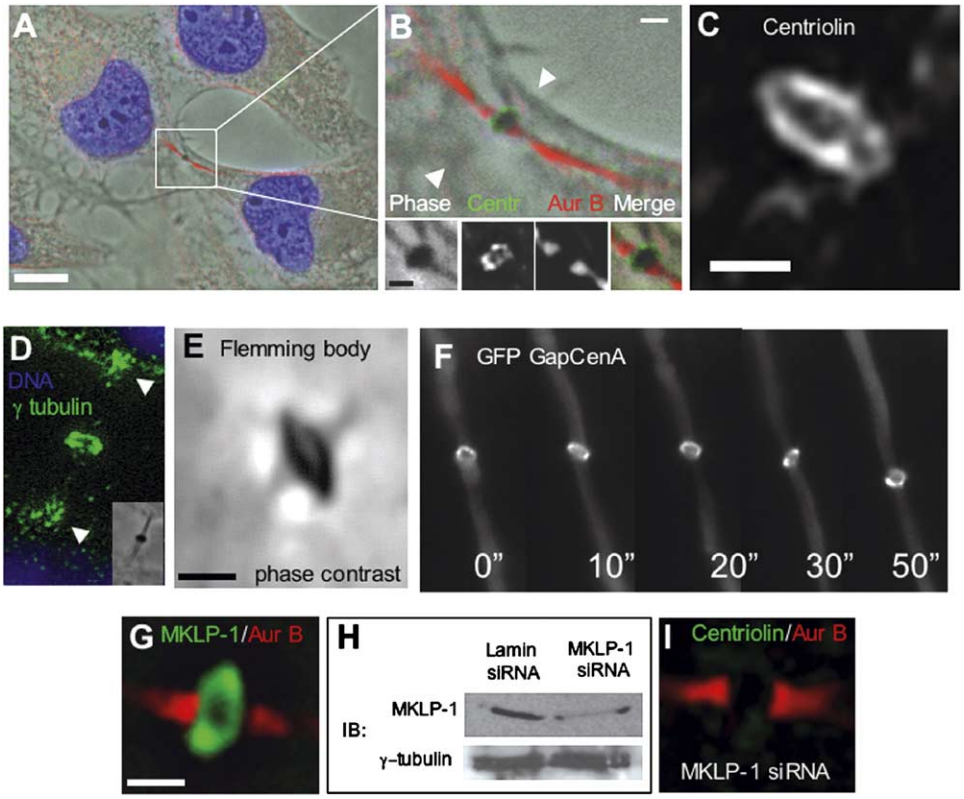


Figure 1. Centriolin Localizes to a Midbody Ring

(A) Immunofluorescence/phase image of HeLa cell during cytokinesis showing the phase-dense Flemming body within the larger diameter of the plasma membrane (arrowheads in [B]).

(B and C). Boxed region enlarged with insets (B) to show the centriolin ring (Centr, enlarged in [C]) as part of the Flemming body (phase) and flanked bilaterally by Aurora B (Aur B).

(D) γ-tubulin localizes to the midbody ring (inset, Flemming body) and sites of presumed microtubule minus ends (arrowheads).

(E) The Flemming body forms a ring.

(F) GFP-tagged GAPCenA localizes to the midbody ring and is highly dynamic (time in s).

(G–I) MKLP-1 localizes to the midbody ring (G) and, upon depletion, mislocalizes centriolin from the midbody (I). Immunoblots (IB) from cells treated with siRNAs targeting MKLP-1 or lamin A/C (control) (H). γ-tubulin, loading control. Scale bars in (A), 10 μm; (B), 5 μm; (C), (E), and (G), 1 μm.

SNARE and exocyst complexes at a unique midbody site and also requires asymmetric transport and fusion of secretory vesicles at this site.

Results

Centriolin Is Part of a Ring-like Structure at the Central Midbody during Cytokinesis

We previously showed that centriolin localized to the midbody during cytokinesis (Gromley et al., 2003). Using high-resolution deconvolution microscopy, we now demonstrate that centriolin is part of a unique ring-like structure within the central portion of the midbody, which we call the midbody ring (observed in ~75% of all telophase cells, Figures 1A–1C). The midbody ring was 1.5–2 μm in diameter (Figure 1C), contained γ-tubulin (Figure 1D), and colocalized with the phase-dense Flemming body (Figure 1B, inset) (Paweletz, 1967). In fact, high-magnification phase-contrast imaging revealed that the Flemming body was organized into a ring-like structure (Figure 1E). The midbody ring was flanked by Aurora B kinase, which colocalized with microtubules on either side of the ring (Figure 1B, inset). Several other proteins localized to the midbody ring including ectopically expressed GFP-GAPCenA, a GTPase-activating protein previously shown to localize to centrosomes (Cuif et al., 1999). Time-lapse imaging of GFP-GAPCenA and other proteins in living cells showed that the midbody ring was dynamic, moving between cells and tipping from side to side to reveal the ring structure (Figure 1F; see also Movie S1 in the Supplemental Data available with this article online). In addition, midbody-ring localization of GFP-GAPCenA confirmed the ring structure seen by immunofluorescence microscopy and demonstrated that there were no antibody penetration problems in this midbody region as seen for other antigens (Saxton and McIntosh, 1987). The midbody ring was distinct from the actomyosin ring and did not change in diameter during cytokinesis (Figures 1A and 1B). It appeared during the early stages of actomyosin-ring constriction and persisted until after cell cleavage (see below).

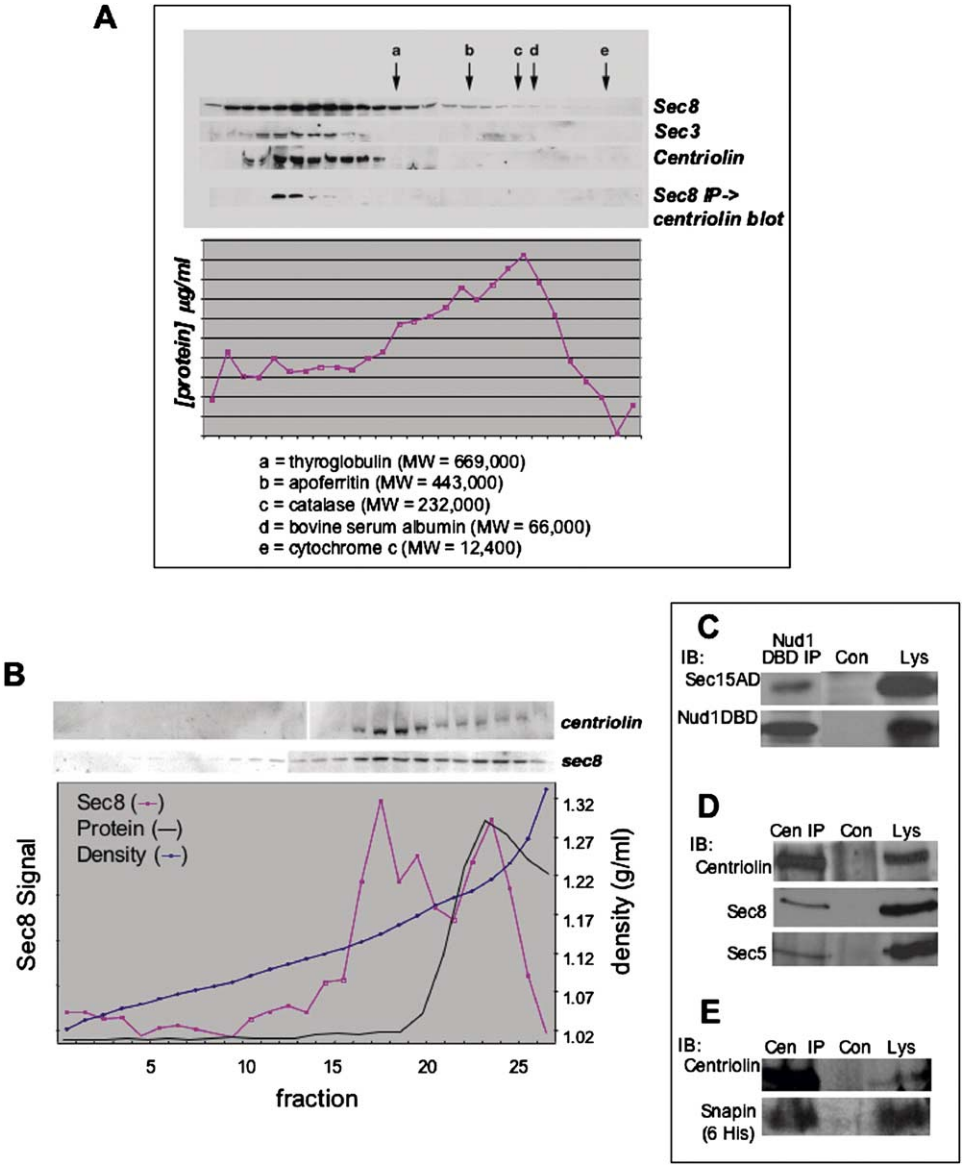


Figure 2. Centriolin Interacts with Exocyst Components and Snapin

(A) Gel filtration (Superose 6) using MDCK cell lysates shows that centriolin coelutes with peak exocyst fractions (top). Immunoprecipitation (IP) of sec8 coprecipitates centriolin. Graph, total protein profile; markers a–e are indicated.

(B) Following isopycnic centrifugation (iodixanol), centriolin comigrates in peak fractions containing sec8 (upper panels). Graph shows sec8 levels, iodixanol density, and total protein.

(C) Immunoprecipitation (IP) of Nud1-DBD (DBD antibody) pulls down sec15-AD (left). DBD, DNA binding domain; AD, activation domain; Con, control beads; Lys, lysate.

(D) Endogenous exocyst components coimmunoprecipitate with endogenous centriolin (Cen IP).

(E) Endogenous centriolin immunoprecipitates (Cen IP) overexpressed His_6-tagged snapin.

The centralspindlin components MKLP-1/CHO1/ZEN-4 (Figure 1G) and MgcRacGAP/CYK-4 (data not shown) also localized to the midbody ring and appeared earlier than centriolin during actomyosin-ring constriction. Depletion of MKLP-1 by RNAi to 18% of control levels (n = 2 experiments) prevented recruitment of centriolin to the ring (Figures 1H and 1I). In contrast, depletion of centriolin had no effect on the localization of MKLP-1 or MgcRacGAP (data not shown). These data suggested that centralspindlin anchored centriolin to the midbody ring.

Centriolin Interacts with the Exocyst Complex and the SNARE-Associated Protein Snapin and Is in Membrane-Associated Cytoplasmic Fractions

To determine the molecular function of centriolin in cytokinesis, we performed a yeast two-hybrid screen using a 120 amino acid domain of centriolin that is required for the cytokinesis function of centriolin and shares homology with budding- and fission-yeast genes (Nud1/Cdc11) involved in cytokinesis and mitotic exit (Gromley et al., 2003). A screen of approximately 12 million clones from a human testis cDNA library

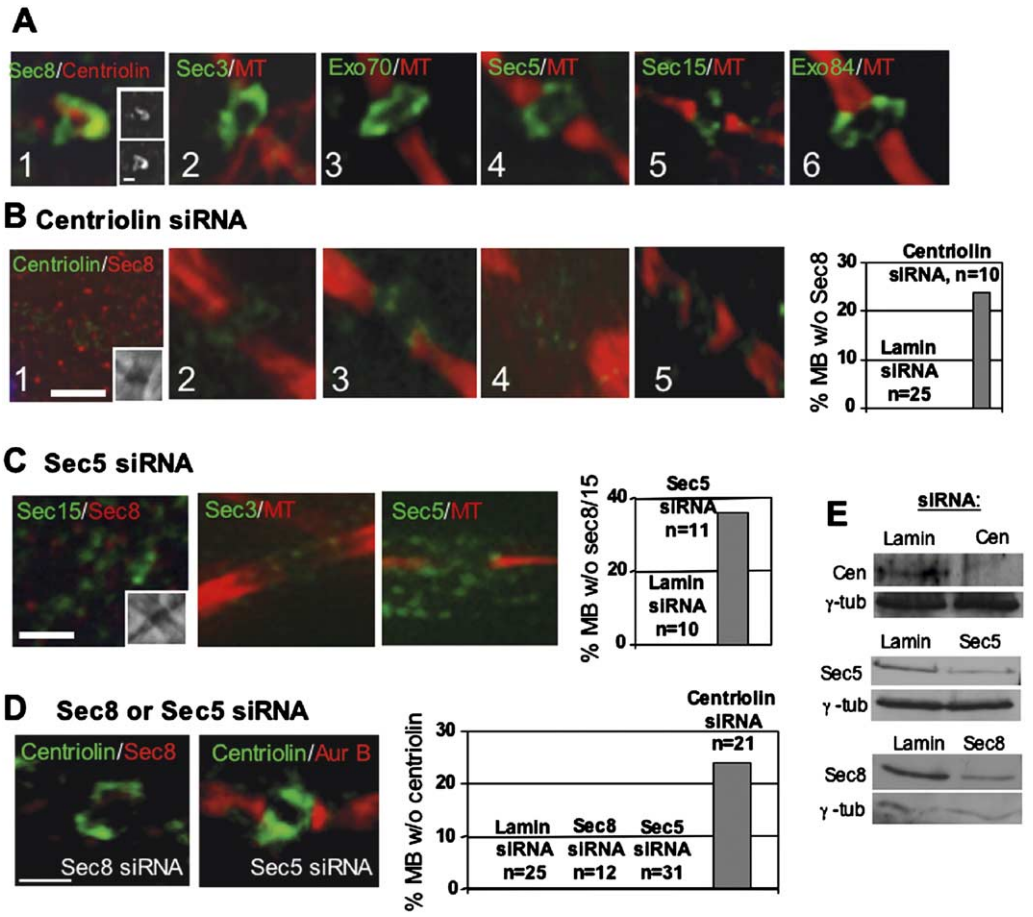


Figure 3. Exocyst Localization to the Midbody Ring Is Centriolin Dependent

(A) Immunofluorescence images of exocyst components (green) costained with centriolin antibodies (panel 1) or with anti-α-tubulin antibody (red) to visualize microtubules (MTs, panels 2–6). Panel 1 inset: top, sec8; bottom, centriolin.

(B) Cells depleted of centriolin lack midbody-associated exocyst. Images labeled as in A1–A5; B1 inset, Flemming body. Graph, percentage of midbodies (MB) without (w/o) sec8 signal following treatment with siRNAs targeting lamin A/C or centriolin; other cells have reduced levels (see text).

(C) siRNA depletion of sec5 disrupts the exocyst from midbodies costained with two exocyst proteins (C1 inset, phase) or one exocyst protein and microtubules (C2–C3). Graph, percentage of midbodies (MB) lacking sec5 staining in cells treated with lamin A/C or sec5 siRNAs.

(D) Exocyst disruption by siRNAs does not affect centriolin midbody localization. Graph, percentage of midbodies (MB) lacking centriolin stain following treatment of indicated siRNAs. Scale bar equals 1 μm (all panels).

(E) Immunoblots showing reduction of proteins targeted by siRNAs. γ-tubulin (γ-tub), loading control. Cen, centriolin.

yielded two potential interacting proteins: sec15, a member of the exocyst complex, and snapin, a SNARE-associated protein.

Additional biochemical analysis confirmed the yeast two-hybrid interactions and demonstrated that centriolin was in a large complex associated with membranes (Figure 2). The centriolin Nud1 domain fused to the DNA binding domain (DBD) and sec15 fused to the activation domain (AD) were coexpressed in the same yeast cells. Immunoprecipitation of the Nud1 fusion protein effectively coprecipitated the sec15 fusion protein (Figure 2C). To test whether other members of the exocyst complex were bound to centriolin, we immunoprecipitated endogenous centriolin from HeLa cell lysates with affinity-purified centriolin antibodies and showed that sec8 and sec5 coprecipitated (Figure 2D). Gel filtration experiments (Superose 6) using MDCK cell lysates demonstrated that centriolin coeluted with fractions containing the exocyst complex (detected with antibodies to sec8 and sec3, Figure 2A). Centriolin was eluted as a single peak that overlapped with peaks of sec3 and sec8. We next asked if centriolin coimmunoprecipitated with the exocyst. Antibodies to sec8 were added to each of the fractions from the gel filtration column, and immune complexes were collected and probed with affinity-purified centriolin antibodies as described (Gromley et al., 2003). Centriolin was found only in fractions containing exocyst components (Figure 2A). The centriolin-containing fractions eluted earlier than the peak of sec 3 or sec8, suggesting that the exocyst fraction to which centriolin was bound was different from the cytosolic and lateral plasma-membrane fractions of the exocyst (Yeaman et al., 2004). The exocyst-centriolin fractions did not cofractionate with the

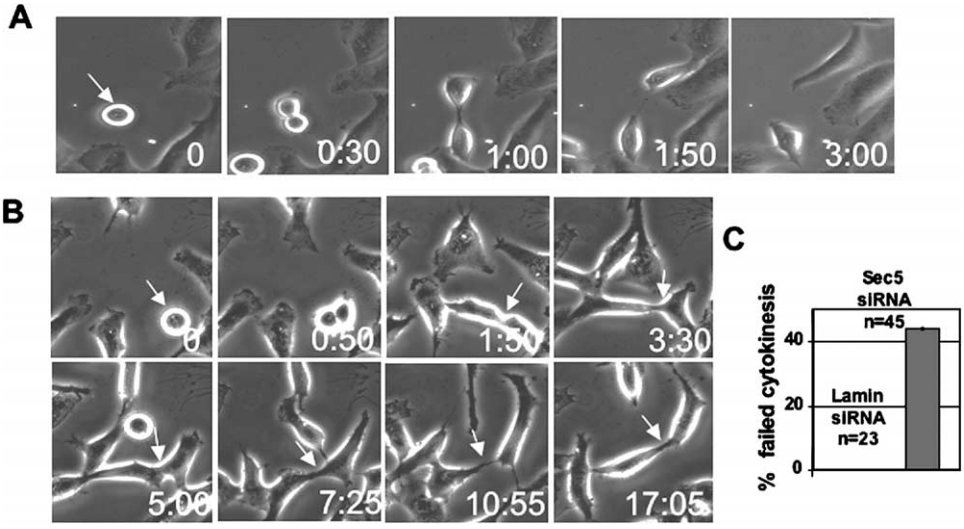


Figure 4. Exocyst Disruption Induces Cytokinesis Defects

(A) Time-lapse images of a HeLa cell treated with lamin A/C siRNAs showing a mitotic cell entering mitosis (arrow), forming a cleavage furrow, and cleaving into two separate cells in 3 hr. Time, hr:min.

(B) A cell depleted of sec5 enters mitosis (arrow), forms a cleavage furrow with normal timing (~50 min), and remains interconnected by a thin intercellular bridge for over 17 hr (panels 1:50 through 17:05).

(C) Graph shows percentage of mitotic cells that fail cytokinesis; many others are delayed (see text).

bulk of the cellular protein and eluted considerably earlier than thyroglobulin (MW 669,000) suggesting it was part of a large complex.

Since the exocyst associates with membrane vesicles, we next tested whether centriolin was also present in membranous fractions. Cell homogenates were prepared in the absence of detergent and underlain at the bottom of linear iodixanol gradients. Isopycnic centrifugation was performed, and fractions were probed for both centriolin and the exocyst component sec8. Centriolin "floated up" to fractions lighter than the cytosol having a buoyant density of $\delta \sim 1.14$ g/ml (Figure 2B). The centriolin peak cofractionated with a major peak of Sec8 that was slightly less dense than the junction-associated peak of Sec8 described previously in confluent MDCK cells ($\delta \sim 1.16$ g/ml; Yeaman et al., 2004). Little to no centriolin was observed at other positions in the gradient or in the major protein peak, suggesting that most if not all centriolin was associated with membranes. Taken together, the density gradient, immunoprecipitation, and chromatography data support the conclusion that centriolin associates with the exocyst in a very large complex bound to cellular membranes. The yeast two-hybrid interaction between centriolin and the low-abundance protein snapin was confirmed by showing that endogenous centriolin coimmunoprecipitated a His_6-tagged snapin fusion protein expressed in HeLa cells (Figure 2E) and by the centriolin-dependent midbody localization of snapin (see below).

The Exocyst Complex Colocalizes with Centriolin at the Midbody Ring

Further support for the centriolin-exocyst interaction was obtained by showing that exocyst-complex components localized to the midbody ring with centriolin. HeLa cells were colabeled with antibodies against one of several exocyst components (sec3, sec5, sec8, sec15, exo70, or exo84) and either microtubules or centriolin (Figure 3A). We found that all these exocyst components localized to the midbody ring during cytokinesis and formed a ring-like structure similar to that seen for centriolin. In fact, double-stained images revealed considerable overlap between sec8 and centriolin, indicating that they were part of the same structure (Figure 3A, panel 1). We also showed that a myc-tagged form of sec8 localized to the midbody ring when expressed in HeLa cells (Figure S1), confirming the localization seen with antibodies directed to the endogenous protein.

Midbody Localization of the Exocyst Is Disrupted in Cells Depleted of Centriolin

We next tested whether centriolin was required for midbody-ring localization of the exocyst. siRNA-mediated depletion of centriolin resulted a in ~70% reduction in centriolin protein levels and complete loss of midbody staining in 24% of cells compared with control cells treated with lamin siRNA (Figures 3B and 3E). Immunofluorescence quantification of midbody signals performed as in our previous studies (Gromley et al., 2003) demonstrated that many of the remaining centriolin-depleted cells had lower levels of midbody staining than controls (48%, n = 23 cells), bringing the total percentage of midbody depleted cells to 72%. Cells that lacked detectable midbody-associated centriolin usually lacked midbody labeling of sec8 (10/10, Figure 3B, panels 1 and 6). Although other exocyst components could not be costained with centriolin because all were detected with rabbit antibodies like centriolin, all were lost from or reduced at midbodies in centriolin-depleted cells (Figure 3B, panels 2–5). For example,

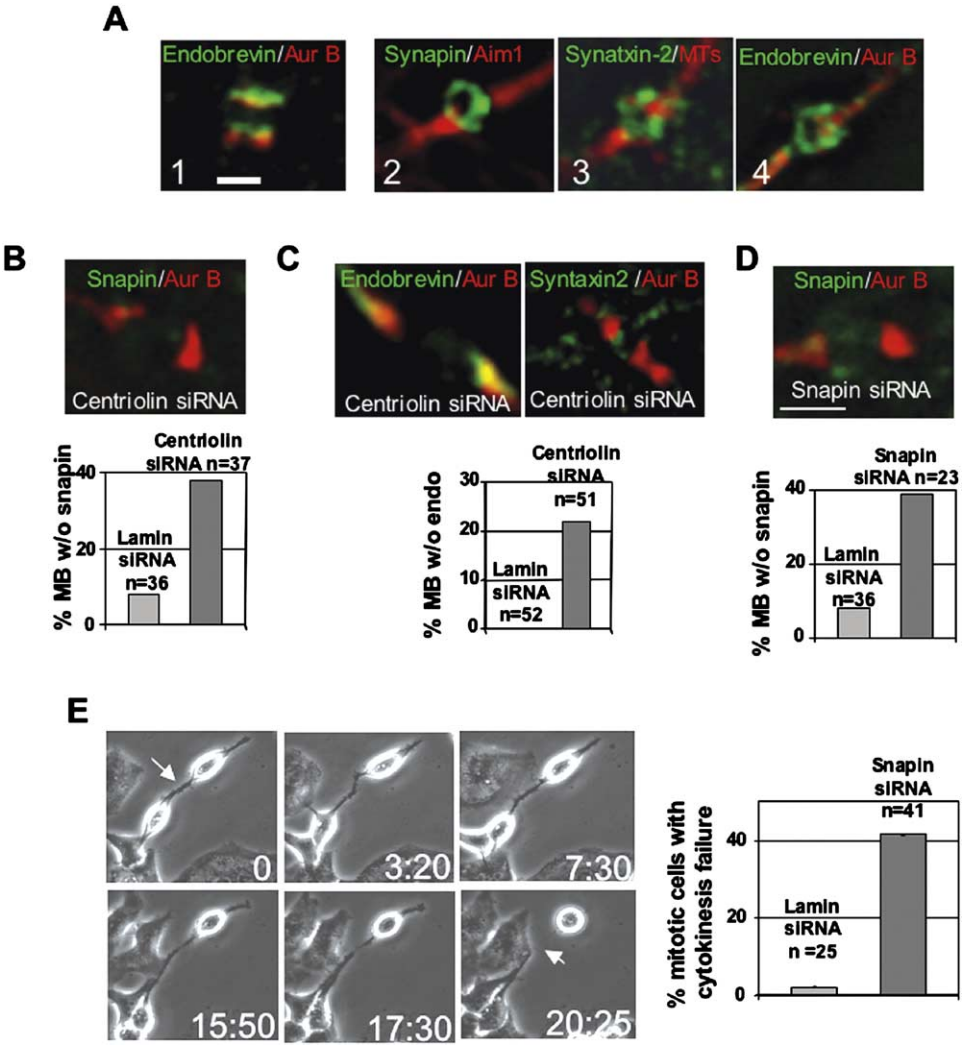


Figure 5. Centriolin siRNA Mislocalizes Midbody-Ring-Associated SNAREs and Snapin, which Disrupts Cytokinesis When Depleted

(A) Endobrevin/VAMP8 (1) localizes adjacent to the midbody ring when snapin is on the ring (2). Later, when the midbody diameter is thin (0.5–1 μm), endobrevin/VAMP8 and syntaxin-2 localize to the ring (3 and 4).

(B) Centriolin-depleted cell shows loss of snapin from the midbody ring (green). Graph, percentage of midbodies lacking snapin after siRNA depletion of proteins.

(C) Centriolin-depleted cells lose SNARE proteins from the midbody ring. Graph, percentage of midbodies lacking endobrevin/VAMP8 staining after indicated siRNAs treatments. Endo, endobrevin.

(D) Snapin-depleted cells show loss of snapin from the midbody ring. Graph, percentage of midbodies lacking snapin after indicated siRNA treatments.

(E) A snapin-depleted cell in cytokinesis (0) remains connected by a thin intercellular bridge for >17 hr before separating (20:25) (time, hr:min). Graph, percentage of mitotic cells that failed cytokinesis.

Exo84 was undetectable at midbodies in 22% of centriolin-depleted cells (n = 9 cells) or had levels below the lowest control midbody staining in 42% of centriolin-depleted cells (n = 19 cells). Significant reduction in midbody staining of centriolin and other exocyst components was observed with a second siRNA targeting a different centriolin sequence (Gromley et al., 2003) (data not shown).

To test whether centriolin was dependent on the exocyst complex for localization to the midbody, we initially targeted sec5 for siRNA depletion. Recent studies showed that mutants of sec5 in *D. melanogaster* disrupted exocyst function (Murthy and Schwarz, 2004) and that RNAi-mediated depletion of sec5 inhibited exocyst-dependent processes in vertebrate cells (Prigent et al., 2003). We found that depletion of sec5 resulted in loss of midbody-associated sec5 as well as other exocyst components, including sec3, sec8, and sec15 (Figures 3C and 3E). These results show that sec5 depletion disrupts midbody-ring localization of the exocyst. In contrast, neither sec5 nor sec8 loss from the midbody affected the association of centriolin with the midbody ring (Figures 3D and 3E). These data demonstrate that centriolin is required for midbody localiza-

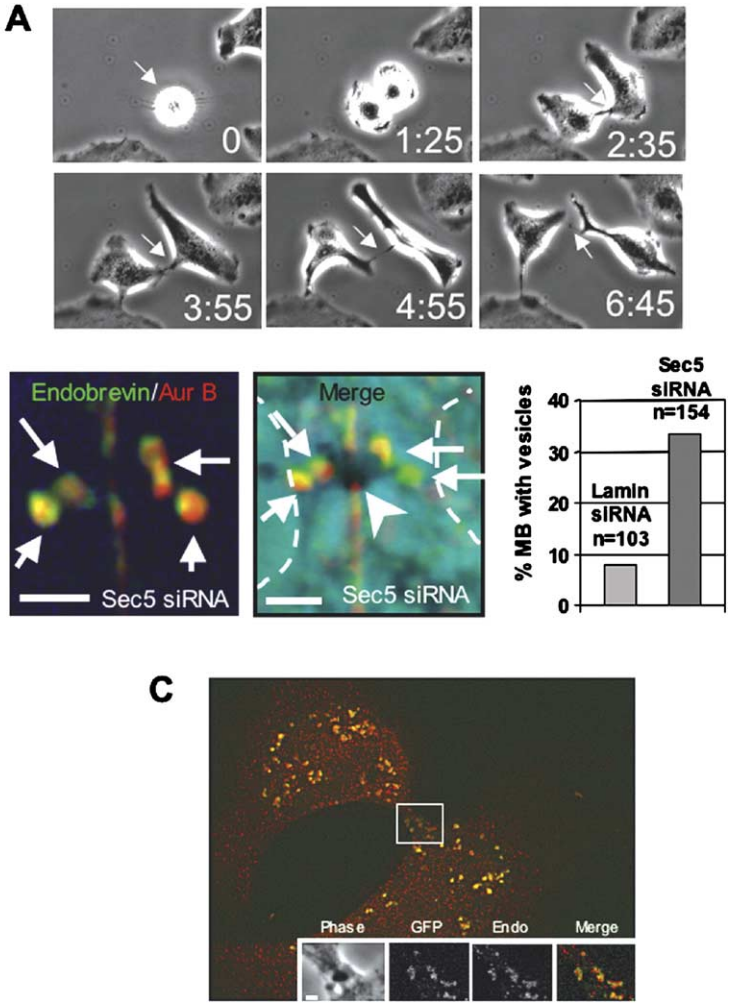


Figure 6. Disruption of the Exocyst Results in Accumulation of Secretory Vesicles at the Midbody Ring

(A) A mitotic cell (0, arrow) treated with BFA exits mitosis and forms a cleavage furrow with normal timing but arrests with a thin intercellular bridge that connects the two daughters (panels 2:35 through 6:45).

(B) sec5 siRNA-treated cells accumulate endobrevin/VAMP8-containing vesicle-like structures (arrows) at the Flemming body (arrowhead, panel 2). Dotted lines, plasma membrane. Graph, percentage of cells with endobrevin/VAMP8 vesicles at the midbody following indicated siRNA treatments. Scale bars, 2 μm.

(C) Endobrevin/VAMP8 (green) localizes to luminal-GFP secretory vesicles (red). Box at midbody is enlarged in insets. Endo, endobrevin/VAMP8.

tion of the exocyst, while localization of centriolin appears to be independent of the exocyst.

Disruption of the Exocyst Causes Failure at the Final Stages of Cytokinesis

Localization of the exocyst to the midbody and its interaction with centriolin suggested that the complex might play a role in cytokinesis. To examine this, we disrupted the midbody-associated exocyst using siRNAs targeting sec5 and examined cytokinesis by time-lapse imaging over a 20 hr time period. We found that over half the cells exhibited severe cytokinesis defects, including failure in the final abscission step (42%, Figures 4B and 4C, Movie S3) and delays during cytokinesis (24%, n = 18) compared with control lamin siRNA-treated cells (Figures 4A and 4C, Movie S2). Some cells remained interconnected by thin cytoplasmic bridges (Figure 4B, panel 17:05 and Movie S3) and sometimes entered one or more additional rounds of mitosis while still connected to their partner cells. Sec5-depleted cells viewed for an additional 24 hr showed a similar level of cytokinesis defects (data not shown), suggesting that nearly all cells in the culture experienced cytokinesis problems over time. Cytokinesis defects were also observed when the exocyst was disrupted by siRNA depletion of sec15 and sec8 (data not shown). Cells remained healthy, as no differences in cell morphology or mitochondrial function were observed. These data show that disruption of the exocyst produces late-stage cytokinesis defects similar to centriolin (Gromley et al., 2003) and demonstrates a requirement for the exocyst in the final stages of animal cell cytokinesis.

Snapin and SNARE Components Localize to the Midbody Ring in a Centriolin-Dependent Manner

Snapin was originally considered to be a neuron-specific protein, but recent studies demonstrated that it is also expressed in nonneuronal cells (Buxton et al., 2003). Snapin may facilitate assembly of SNARE complexes and may define a limiting step in vesicle fusion mediated by PKA phosphorylation (Chheda et al., 2001). Although the role of snapin in neurotransmission has been questioned (Vites et al., 2004), recent results indicate that it is essential for this process (Thakur et al., 2004). The role of snapin in cytokinesis is currently unknown. Using previously characterized antibodies to snapin (Thakur et al., 2004), we demonstrated that the

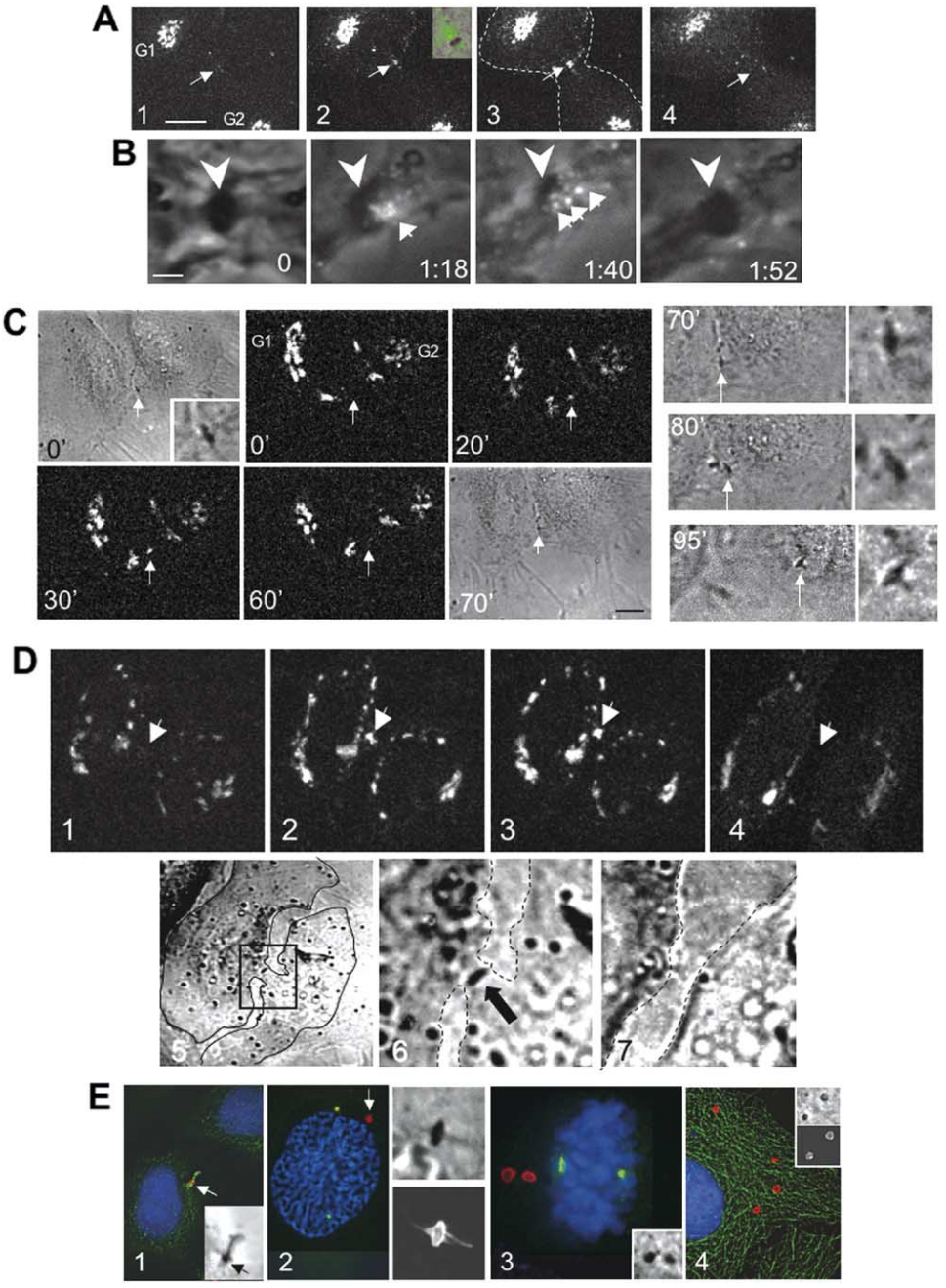


Figure 7. Asymmetric Delivery of Secretory Vesicles to One Side of the Flemming Body Is Followed by Abscission at This Site

(A) A dividing HeLa cell expressing luminal GFP accumulates secretory vesicles on one side of the Flemming body (arrows in 2 and 3, inset). In panel 1, most luminal-GFP signal is in Golgi complexes (G1 and G2). The signal appears transiently at one side of the midbody (2 and 3, arrows; Movie S5) and is lost, although Golgi signal remains (4). Scale bar in panel 1, 10 μm.

(B) Higher-magnification images of another cell (see Movie S6) showing unidirectional delivery of luminal-GFP-containing vesicles from one nascent daughter cell to one side of the Flemming body (arrowhead). GFP vesicles move to the Flemming body from the cell on the right (1:18 and 1:40, arrows; see Movie S6) and quickly disappear (1:52), presumably due to vesicle fusion with the plasma membrane and diffusion of the signal into the extracellular space. Phase and GFP signals are overlaid. Time, hr:min. Scale bar in panel 1, 1 μm.

(C) Lum-GFP vesicle delivery to the Flemming body (0'–30', arrows) followed by signal loss (60', at arrow) and abscission (80' and 95'). Phase-contrast images were taken after disappearance of GFP signal. Enlargements of Flemming body are shown to the right of each low-magnification image in 70'–95'. Scale bar at 70': 10 μm for 0'–95' and 2 μm for enlargements in 70'–95'.

(D) Lum-GFP vesicle delivery to one side of the midbody (panels 1–3) followed by disappearance of the GFP signal (panel 4) and abscission (loss of intercellular bridge, panels 5–7, arrows). The box in panel 5 is enlarged in panel 6. Solid and dotted lines show cell boundaries.

(E) Postmitotic cell (1) showing microtubules (green, GT335 antibody) of the intercellular bridge (phase-contrast image, inset) attached to one of the two daughter cells; no detectable midbody microtubules are seen on the other cell. Microtubules are on both sides of the midbody ring (arrow, red, MKLP-1) and Flemming body (inset, phase), showing that the midbody with attached microtubules was delivered to one daughter cell. Prophase HeLa cell (2) with condensing chromatin (blue) and two centrosomes (green) has a midbody ring and lateral material

protein localized to the midbody ring at the same time as the exocyst and shortly after centriolin (Figure 5A, panel 2).

Previous immunofluorescence studies showed that the v-SNARE endobrevin/VAMP8 and t-SNARE syntaxin-2 were enriched in the region of the midbody flanking the Flemming body and coincident with microtubules and Aurora B staining (Low et al., 2003). Using the same antibodies, we confirmed the localization pattern of endobrevin/VAMP8 (Figure 5A, panel 1) and syntaxin-2 (data not shown). Very late in cytokinesis, the intercellular bridge narrows to ~0.5 μm, and microtubule bundles are reduced in diameter to 0.2–0.5 μm. At this time, endobrevin/VAMP8 and syntaxin-2 joined centriolin, snapin, and the exocyst at the midbody ring (Figure 5A, panels 3 and 4). siRNA depletion of centriolin eliminated the midbody-ring localization of snapin (>35% of cells, Figure 5B), endobrevin/VAMP8 (>20% of cells, Figure 5C), and syntaxin-2 (Figure 5C). Of the remaining cells, 24% and 36% showed midbody staining levels below those of controls for snapin (n = 22) and endobrevin/VAMP8 (n = 25), respectively. As shown earlier, midbody-ring integrity was not compromised under these conditions, as MKLP-1 and MgcRacGAP remained at this site in cells with reduced centriolin. These results indicated that centriolin was required for midbody-ring localization of v- and t-SNARE proteins and the SNARE-associated protein snapin.

Snapin Depletion Mislocalizes the Protein from the Midbody and Induces Cytokinesis Defects

Midbodies in 41% of snapin-depleted cells showed no detectable snapin staining (Figure 5D). Time-lapse imaging over a 22 hr period showed that 40% of snapin-depleted cells experienced late-stage cytokinesis failure (Figure 5E, Movie S4). Other cells showed long delays and often remained connected by a thin intercellular bridge (data not shown). When cultures were imaged for an additional 24 hr, we observed multicellular syncytia resulting from multiple incomplete divisions and additional individual cells undergoing cytokinesis failure. This suggested that most cells in the population ultimately failed cytokinesis and that some failed multiple times. Occasionally, cells separated when one of the attached daughters re-entered mitosis, possibly due to tensile forces generated by cell rounding during mitosis (Figure 5E, Movie S4). These results demonstrated that snapin was necessary for abscission and suggested that it functioned by anchoring SNARE complexes at the midbody.

Disruption of the Exocyst Results in Accumulation of Secretory Vesicles at the Midbody

We next tested whether the late-stage cytokinesis defects observed in this study resulted from changes in membrane trafficking to the midbody. As a first test of this idea, we used brefeldin A, which disrupts cytokinesis in *C. elegans* presumably due to inhibition of post-Golgi secretory-vesicle trafficking (Skop et al., 2001). In HeLa cells treated with brefeldin A, we observed late-stage cytokinesis defects (Figure 6A) that were similar to those observed following depletion of centriolin. Many cells were delayed in or failed cytokinesis (n = 9/13 cells in two separate experiments). This suggested that post-Golgi vesicle trafficking was involved in late-stage cytokinesis events in vertebrate cells, although brefeldin A is known to affect other membrane-trafficking pathways (Antonin et al., 2000).

Based on the localization of the exocyst to the midbody ring, we reasoned that the vesicle-tethering function of the complex might be operating at this site to facilitate fusion of v-SNARE-containing vesicles at the late stages of cytokinesis. To test this idea, we depleted cells of sec5 to disrupt exocyst complexes and examined the localization of v-SNARE (endobrevin/VAMP8) containing vesicles. We observed a collection of small, spherical endobrevin/VAMP8-containing structures resembling vesicles at the midbody (Figure 6B, panel 1, arrows) that were positioned around the phase-dense Flemming body (Figure 6B, arrowhead, panel 2). Although these structures were occasionally seen in control lamin A/C siRNA-treated cells, they were significantly increased in sec5-depleted cells (Figure 6B, graph).

To determine whether the endobrevin/VAMP8-containing structures were secretory vesicles, we used a more specific marker for the secretory pathway. We expressed a GFP-tagged construct containing an amino-terminal signal peptide that targets the protein to the lumen of the ER (lum-GFP) (Blum et al., 2000) and lacks retention and retrieval motifs, so it would not be expected to target to endosomes, multivesicular bodies, or lysosomes. The lum-GFP was efficiently secreted from nondividing MDCK cells following a 19°C *trans*-Golgi network block and release from the block in the presence of protein-synthesis inhibitors (C.Y., unpublished data). When we expressed lum-GFP, numerous GFP-containing vesicles were observed in the cytoplasm. Following fixation and staining for endobrevin/VAMP8, we found that most of the endobrevin/VAMP8 vesicles colabeled with lum-GFP throughout the cytoplasm (Figure 6C) and within the intercellular bridge during late stages of cytokinesis (Figure 6C, insets). This observation demonstrates that the v-SNARE-containing vesicles that accumulated following disruption of the exocyst are secretory vesicles, an observation similar to that seen in studies in exocyst mutants of *S. cerevisiae* where vesicles dock normally but fail to fuse with the plasma membrane (Guo et al., 2000)

Asymmetric Delivery of Secretory Vesicles to the Midbody Is Followed by Abscission

At early stages of cytokinesis, we observed numerous GFP-labeled secretory vesicles in Golgi complexes and

stained with MKLP-1 (arrow, red) and in enlargement (bottom right); the Flemming body with flanking material is enlarged at upper right. Metaphase cell (3) with two midbody rings stained for MKLP-1 (red). Inset, two Flemming bodies corresponding to the two MKLP-1-stained structures. Centrosomes, green; DNA, blue. Interphase cell (4) showing four MKLP-1-stained midbody rings (red). Two are enlarged in lower inset and colocalize with phase-dense Flemming bodies (upper inset). DNA, blue; microtubules, green.

cell bodies of nascent daughter cells but few within intercellular bridges (Figure 7A, panel 1). However, at a late stage of cytokinesis when the intercellular bridge narrowed to a diameter of ~2 μm and the midbody microtubule bundle was reduced to a diameter of 0.5–1 μm, GFP secretory vesicles accumulated in the intercellular bridge near the midbody ring (Figure 7A, Movie S5). Higher-magnification imaging of another cell at a similar cell-cycle stage revealed labeled secretory vesicles moving suddenly and rapidly (within 20 min) from the cell bodies into the intercellular bridge and up to the midbody ring (Figure 7B, Movie S6). In 11/11 cells, the vesicles were delivered primarily if not exclusively from one of the nascent daughter cells (Figure 7B, center panels). Vesicles packed into the region adjacent to the phase-dense Flemming body (Figure 7B, panels 2 and 3, large arrowhead; Movie S6). Within 20 min, the GFP signal disappeared (Figure 7B, last panel and Figure 7A, last panel), suggesting that the vesicles fused with the plasma membrane, releasing the GFP signal into the extracellular space where it was free to diffuse. Loss of the GFP signal was not due to photobleaching because GFP-labeled vesicles in cell bodies adjacent to the intercellular bridge and in the Golgi complex retained the signal. We next examined the relationship between vesicle delivery to the midbody and abscission. We found that, shortly after the GFP signal was lost from the midbody region, the cell cleaved on the side of the Flemming body that received the GFP vesicles (6/6 cells from four experiments, Figure 7C). The cell on the opposite side received the Flemming body (Figure 7C, 70'–95' and Figure 7D). In some cases, the Flemming body moved around rapidly after abscission on the cell surface (Movie S7), suggesting that the structure was not anchored at a discrete point on the new daughter cell. Postdivision midbodies contained multiple midbody-ring components and retained microtubules from both sides of the midbody ring (Figure 7E, panel 1). They persisted for some time after abscission, consistent with previous results (Mishima et al., 2002), and were often present in multiple copies, suggesting that they were retained through several cell cycles (Figure 7E, panels 2–4). These structures were seen on ~35% of HeLa cells and often retained features of the Flemming body and midbody ring, including MKLP-1 staining, Aurora B staining, phase-dense Flemming bodies, and localization to the plasma membrane (Figure 7E, data not shown). This suggested that supernumerary midbodies represent structures from previous divisions similar to the bud scars observed in yeast (Chen and Contreras, 2004).

Discussion

A Model for the Final Stage of Cytokinesis

This study defines several distinct molecular and structural steps during the late stages of cytokinesis (Figure 8). During cleavage-furrow ingression, MKLP-1 and MgcRacGAP arrive at the midbody ring (Figure 8A). When the intercellular bridge forms, centriolin localizes to the ring (Figure 8B), followed by snapin and exocyst proteins (Figure 8C). When the diameter of the midbody microtubule bundle and the intercellular bridge are reduced to ~0.5–1 μm, endobrevin/VAMP8 (v-SNARE) and syntaxin-2 (t-SNARE) move to the midbody ring. The v-SNAREs are part of secretory vesicles that move asymmetrically into the intercellular bridge predominantly from one nascent daughter cell; binding to v-SNAREs may incorporate t-SNAREs into this organization. The vesicles pack into the area adjacent to the ring and appear to fuse, releasing their contents into the extracellular space (lum-GFP, Figures 8D and 8E). Vesicle fusion with the plasma membrane may be initiated near the midbody ring where v- and t-SNAREs are localized. This could be followed by additional fusion events between vesicles and the plasma membrane as well as vesicle-vesicle fusion events (homotypic) mediated by SNAP23/25, a v-SNARE involved in compound exocytosis (Takahashi et al., 2004) (Figures 8F and 8G). Abscission then occurs at the site of vesicle fusion, and the entire midbody remains with the daughter cell opposite the fusion site (Figure 8H). Abscission could be triggered by arrival of v- and t-SNAREs at the midbody ring; release of SNAP23/25 from lipid rafts (Takahashi et al., 2004; Takeda et al., 2004); phosphorylation of snapin by PKA, which mediates its binding to the t-SNARE complex (Buxton et al., 2003; Chheda et al., 2001); or another event. Dynamic movement of the postabscission midbody ring suggests connections to motile forces within the cell, although this remains to be determined.

Asymmetric Delivery of Secretory Vesicles Marks the Site of Abscission

It is remarkable that secretory vesicles loaded with luminal GFP move into the intercellular bridge from only one of the two prospective daughter cells. The mechanism of this asymmetric vesicle delivery is unknown. It is tempting to speculate that a signal, negative or positive, emanates asymmetrically from one centrosome in the dividing cell. Centrosomes in the two prospective daughter cells are different in that one was "born" from the older centriole in the previous cell division during the centrosome duplication process (Doxsey, 2001). Consistent with this idea is the asymmetric spindle-pole body (SPB) localization of budding- and fission-yeast proteins that control mitotic exit and cytokinesis (Doxsey et al., 2005; Grallert et al., 2004; Molk et al., 2004). In *S. pombe*, inhibitors of mitotic exit (Cdc16p and Byr4p) localize to the "old" SPB while activators of mitotic exit (Cdc7p and presumably Sid1p and Cdc14p) localize to the new SPB (Grallert et al., 2004). The relevance of this localization in both yeasts is still unknown. Further studies will be required to determine the role of centrosome protein asymmetry in the unidirectional delivery of secretory vesicles and abscission in animal cells. It has been suggested that the mother centriole moves to the intercellular bridge in telophase cells to coordinate the final steps in cytokinesis (Piel et al., 2001), although this was not consistently observed in this study (data not shown) or another that investigated several cell lines (RPE-1, Ptk-1, CV-1, NRK-52E; A. Khodjakov, personal communication).

The final stages of cytokinesis in animal cells share features with cell division in higher plants. Higher plant cells cannot divide using an actomyosin-based cleavage furrow due to the presence of a nonpliant cell wall,

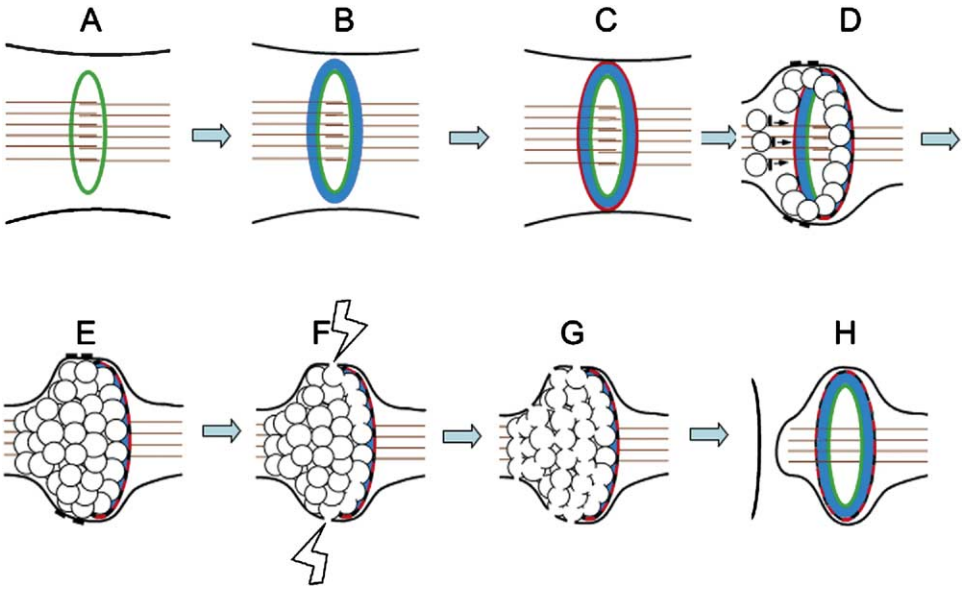


Figure 8. Model Depicting Vesicle-Mediated Abscission during Cytokinesis

(See text for details.)

(A) MKLP-1 and MgcRacGAP (green) arrive at midbody ring after cleavage furrowing has progressed. Microtubules, brown; plasma membrane, upper and lower lines.

(B and C) Centriolin moves to ring ([B], blue) and anchors sec15, other exocyst components, and snapin ([C], red).

(D) When midbody microtubules are reduced and the membrane constricted, v- and t-SNAREs ([D], black) move to the midbody ring from one prospective daughter cell. v-SNAREs presumably move with vesicles and bind there in a centriolin-dependent manner; t-SNAREs on the plasma membrane could bind through v-SNAREs.

(E) Vesicles heterogeneous in diameter pack asymmetrically into the intercellular bridge adjacent to the midbody ring.

(F and G) Vesicles adjacent to the ring containing SNARES and exocyst fuse with the plasma membrane (F) as well as at other plasma-membrane sites and with one another (G).

(H) Abscission follows at the site of membrane fusion, and the midbody is retained by the daughter cell opposite the fusion site. The released midbody ring contains multiple midbody-ring proteins and usually retains microtubule bundles from both sides of the ring. (In this model, the apparent "layering" of components is a simplification to depict arrival of different components at the midbody.)

so they accomplish cell division by constructing a new membrane at the division plane, called the cell plate, that is independent of the plasma membrane and is established by microtubule-dependent delivery and fusion of vesicles at this site (Albertson et al., 2005; Finger and White, 2002; Jurgens, 2005). Our data show that the coordinated delivery of vesicles to the midbody ring during the late stages of cytokinesis is also required for the final stages of cell division in animal cells. However, we still do not understand the mechanism of secretory-vesicle delivery to the midbody, the role of microtubules in this process, or the precise contribution of vesicle transport and fusion to abscission. The presence of vesicles with heterogeneous diameters adjacent to the midbody ring prior to abscission is consistent with a model in which some vesicles fuse together prior to fusion with the plasma membrane. This would be analogous to the cell plate in plant cells. The enodocytic pathway also appears to play a role in cell cleavage as components (dynamin, FIP3, Rab11) and compartments (endosomes) involved in this pathway affect the late stages of cytokinesis (Thompson et al., 2002; Wilson et al., 2005). Recycling endosomes have been shown to move from both prospective daughter cells to the midbody during cytokinesis then return to the daughter-cell cytoplasm (Wilson et al., 2005). It is still unclear how recycling endosomes participate in abscission and how the bidirectional movement of endosomes into the intercellular bridge is related to the unidirectional movement of secretory vesicles to this site in our study.

Structure and Persistence of the Midbody Ring

We have shown that many proteins localize to the midbody ring and that the phase-dense Flemming body is also organized into the shape of a ring. This is consistent with earlier ultrastructural studies that describe cytoplasmic channels coursing through the central midbody (Mullins and Biesele, 1977). The ring structure bears a resemblance to bud scars of *S. cerevisiae,* which serve as markers for longevity (Chen and Contreras, 2004). The midbody ring in animal cells is inherited by the daughter cell that lies opposite the site of vesicle delivery and appears to persist, as it is often seen in mitotic cells prior to cytokinesis and found in multiple copies in interphase cells (Figure 7E) (Mishima et al., 2002). Shortly after abscission, the midbody ring contains microtubules that extend from both sides of the ring. This suggests that dissolution of microtubule bundles adjacent to the midbody ring is not an absolute requirement for the final stage of cytokinesis but rather that abscission can result in transfer of the entire mid-

body and associated microtubules into one daughter cell.

Experimental Procedures

Cell Culture and Transfections

The cells used primarily in this study were diploid, telomerase-immortalized human RPE cells (hTERT-RPE-1s, Clontech Laboratories, Inc.) (Morales et al., 1999) and HeLa cells. All cells were grown as previously described (American Type Culture Collection). HeLa cells were transfected as previously described (Lipofectamine, Invitrogen).

Immunofluorescence

Cells were prepared for immunofluorescence, imaged, and deconvolved (Metamorph, Universal Imaging Corp.) using either formaldehyde, formaldehyde followed by methanol, or methanol alone as previously described (Dictenberg et al., 1998). All immunofluorescence images are two-dimensional projections of three-dimensional reconstructions to ensure that all stained material was visible in two-dimensional images. Quantification of signals produced by immunofluorescence staining for various midbody antigens was performed as described for centrosome protein quantification in our earlier studies (Gromley et al., 2003).

Antibodies

Antibodies to the following proteins were used: sec3, sec5, sec8, sec10, exo70, exo84, and sec15 (Yeaman, 2003); centriolin (Gromley et al., 2003); α-tubulin, γ-tubulin, α-His_6, and α-myc (Sigma-Aldrich); Aurora B (Transduction Laboratories); MKLP-1, GAL4 transactivation domain (AD), and GAL4 DNA binding domain (DBD) (Santa Cruz Biotechnology, Inc.); and GT335 for stabilized microtubules (Gromley et al., 2003).

Yeast Two-Hybrid Screen

Yeast two-hybrid library screens were performed following the manufacturer's instructions using a human testis Matchmaker Pre-Transformed Two-Hybrid Library (Matchmaker GAL4 Yeast Two-Hybrid System, Clontech). False positives were eliminated by mating each clone with strains expressing either lamin C or the DNA binding domain alone and plating on quadruple dropout media.

siRNAs

Two siRNAs targeting centriolin and one targeting lamin A/C were used as described (Gromley et al., 2003). Additional siRNAs targeted nucleotides in the following proteins: MKLP-1 (189–207), sec5 (260–278), sec8 (609–627), and snapin (312–330). Cells were examined 24–48 hr after siRNA treatment. siRNAs were used at 10–50 nM, and Lipofectamine was the delivery agent (Gromley et al., 2003).

Brefeldin A Treatment

HeLa cells were treated with 5–10 μg/ml brefeldin A (Sigma-Aldrich) and imaged.

Immunoprecipitations

Antibodies to centriolin or exocyst were added to hTERT-RPE cell extracts and incubated at 4°C overnight. The lysis buffer included 50 mM Tris-HCl (pH 7.5), 10 mM Na_2HPO_4 (pH 7.2), 1 mM EDTA, 150 mM NaCl, 1% IGEPAL CA-1630, and protease inhibitors (Mini tablets, Roche Diagnostics, Mannheim, Germany). Superose 6 samples were incubated with antibodies to sec3 and sec8, bound to protein A/G beads (Santa Cruz Biotechnology, Inc.) at 4°C for 2 hr (Yeaman, 2003), and exposed to SDS-PAGE and immunoblotting (Harlow and Lane, 1988).

Time-Lapse Imaging

Time-lapse imaging of cytokinesis was performed using a wide-field microscope (Gromley et al., 2003), and images were taken every 5 min for 18–24 hr. For luminal-GFP-expressing cells (Figure 7B), two concurrent time-lapse programs were used (GFP, phase contrast), and images were taken every 2 min for 3–4 hr. A PerkinElmer spinning-disc confocal microscope with an UltraVIEW CSU-10 head was used for Figures 7A, 7C, and 7D. Images were taken every 5 min and captured on an ORCA-AG cooled CCD camera. Images of GFP-GAPCenA-expressing cells were taken every 10 min on a Zeiss Axiophot microscope equipped with a Hamamatsu digital camera. Mitochondria function was assessed by Mitotracker staining (Molecular Probes).

Exocyst Fractionation

For isopycnic centrifugation, membrane compartments containing exocyst fractions were prepared as described (Grindstaff et al., 1998; Yeaman, 2003). For size-exclusion chromatography, cells were extracted with MEBC buffer (0.5% Nonidet P-40, 50 mM Tris-HCl [pH 7.5], 100 mM NaCl) containing protease inhibitors (0.1 mM Na_3VO_4; 50 mM NaF; 1 mM Pefabloc [Boehringer Mannheim]; and 10 μg/ml each of leupeptin, antipain, chymostatin, and pepstatin A) for 10 min at 4°C. Lysates were first sedimented in a Microfuge (Beckman Instruments, Fullerton, California) for 10 min and then for 30 min at 100,000 × g, passed through a 0.22 μm filter (Millipore), and loaded on a Superose 6 HR 10/30 column (200 μl, 10 mm × 30 cm; Pharmacia Biotech, Inc.) equilibrated in MEBC buffer and 1 mM dithiothreitol with 0.1 mM Pefabloc. Proteins were eluted (0.3 ml/min) at 17°C in 0.5 ml fractions, the concentration of protein in the fractions was determined, and the fractions were used for various assays (fractions 7–30).

Supplemental Data

Supplemental Data include one figure and seven movies and can be found with this article online at http://www.cell.com/cgi/content/full/123/1/75/DC1/.

Acknowledgments

We thank Dan McCollum, Yu-Li Wang, Ted Salmon, and Bill Theurkauf for useful discussions. We thank Z. Sheng (NINDS, NIH) (snapin), T. Wiembs (Lerner Institute, Cleveland) (endobrevin/VAMP8 and syntaxin-2) and M. Glotzer (IMP, Vienna) (MKLP-1, MgcRacGAP) for antibodies. This work was supported in part by grants from the National Institutes of Health to S.J.D. (GM51994) and from the Department of Defense to C.Y. (DAMD-17-03-1-0187).

Received: August 18, 2004
Revised: June 3, 2005
Accepted: July 27, 2005
Published: October 6, 2005

References

Albertson, R., Riggs, B., and Sullivan, W. (2005). Membrane traffic: a driving force in cytokinesis. Trends Cell Biol. *15*, 92–101.

Antonin, W., Holroyd, C., Tikkanen, R., Honing, S., and Jahn, R. (2000). The R-SNARE endobrevin/VAMP-8 mediates homotypic fusion of early endosomes and late endosomes. Mol. Biol. Cell *11*, 3289–3298.

Blum, R., Stephens, D.J., and Schulz, I. (2000). Lumenal targeted GFP, used as a marker of soluble cargo, visualises rapid ERGIC to Golgi traffic by a tubulo-vesicular network. J. Cell Sci. *113*, 3151–3159.

Buxton, P., Zhang, X.M., Walsh, B., Sriratana, A., Schenberg, I., Manickam, E., and Rowe, T. (2003). Identification and characterization of Snapin as a ubiquitously expressed SNARE-binding protein that interacts with SNAP23 in non-neuronal cells. Biochem. J. *375*, 433–440.

Chen, C., and Contreras, R. (2004). The bud scar-based screening system for hunting human genes extending life span. Ann. N Y Acad. Sci. *1019*, 355–359.

Chheda, M.G., Ashery, U., Thakur, P., Rettig, J., and Sheng, Z.H. (2001). Phosphorylation of Snapin by PKA modulates its interaction with the SNARE complex. Nat. Cell Biol. *3*, 331–338.

Cuif, M.H., Possmayer, F., Zander, H., Bordes, N., Jollivet, F., Couedel-Courteille, A., Janoueix-Lerosey, I., Langsley, G., Bornens, M., and Goud, B. (1999). Characterization of GAPCenA, a GTPase

activating protein for Rab6, part of which associates with the centrosome. EMBO J. *18*, 1772–1782.

Dictenberg, J., Zimmerman, W., Sparks, C., Young, A., Vidair, C., Zheng, Y., Carrington, W., Fay, F., and Doxsey, S.J. (1998). Pericentrin and gamma tubulin form a protein complex and are organized into a novel lattice at the centrosome. J. Cell Biol. *141*, 163–174.

Dobbelaere, J., and Barral, Y. (2004). Spatial coordination of cytokinetic events by compartmentalization of the cell cortex. Science *305*, 393–396.

Doxsey, S. (2001). Re-evaluating centrosome function. Nat. Rev. Mol. Cell Biol. *2*, 688–698.

Doxsey, S., McCollum, D., and Theurkauf, W. (2005). Centrosomes in cellular regulation. Annu. Rev. Cell Dev. Biol. *21*, 688–698. in press.

Echard, A., Hickson, G.R., Foley, E., and O'Farrell, P.H. (2004). Terminal cytokinesis events uncovered after an RNAi screen. Curr. Biol. *14*, 1685–1693.

Finger, F.P., and White, J.G. (2002). Fusion and fission: membrane trafficking in animal cytokinesis. Cell *108*, 727–730.

Finger, F.P., Hughes, T.E., and Novick, P. (1998). Sec3p is a spatial landmark for polarized secretion in budding yeast. Cell *92*, 559–571.

Glotzer, M. (2001). Animal cell cytokinesis. Annu. Rev. Cell Dev. Biol. *17*, 351–386.

Glotzer, M. (2005). The molecular requirements for cytokinesis. Science *307*, 1735–1739.

Grallert, A., Krapp, A., Bagley, S., Simanis, V., and Hagan, I.M. (2004). Recruitment of NIMA kinase shows that maturation of the S. pombe spindle-pole body occurs over consecutive cell cycles and reveals a role for NIMA in modulating SIN activity. Genes Dev. *18*, 1007–1021.

Grindstaff, K.K., Yeaman, C., Anandasabapathy, N., Hsu, S.C., Rodriguez-Boulan, E., Scheller, R.H., and Nelson, W.J. (1998). Sec6/8 complex is recruited to cell-cell contacts and specifies transport vesicle delivery to the basal-lateral membrane in epithelial cells. Cell *93*, 731–740.

Gromley, A., Jurczyk, A., Sillibourne, J., Halilovic, E., Mogensen, M., Groisman, I., Blomberg, M., and Doxsey, S. (2003). A novel human protein of the maternal centriole is required for the final stages of cytokinesis and entry into S phase. J. Cell Biol. *161*, 535–545.

Guertin, D.A., Trautmann, S., and McCollum, D. (2002). Cytokinesis in eukaryotes. Microbiol. Mol. Biol. Rev. *66*, 155–178.

Guo, W., Sacher, M., Barrowman, J., Ferro-Novick, S., and Novick, P. (2000). Protein complexes in transport vesicle targeting. Trends Cell Biol. *10*, 251–255.

Harlow, E., and Lane, D. (1988). Antibodies: A Laboratory Manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Jurgens, G. (2005). Plant cytokinesis: fission by fusion. Trends Cell Biol. *15*, 277–283.

Low, S.H., Li, X., Miura, M., Kudo, N., Quinones, B., and Weimbs, T. (2003). Syntaxin 2 and endobrevin are required for the terminal step of cytokinesis in mammalian cells. Dev. Cell *4*, 753–759.

Mishima, M., Kaitna, S., and Glotzer, M. (2002). Central spindle assembly and cytokinesis require a kinesin-like protein/RhoGAP complex with microtubule bundling activity. Dev. Cell *2*, 41–54.

Molk, J.N., Schuyler, S.C., Liu, J.Y., Evans, J.G., Salmon, E.D., Pellman, D., and Bloom, K. (2004). The differential roles of budding yeast Tem1p, Cdc15p, and Bub2p protein dynamics in mitotic exit. Mol. Biol. Cell *15*, 1519–1532.

Mondesert, G., Clarke, D.J., and Reed, S.I. (1997). Identification of genes controlling growth polarity in the budding yeast Saccharomyces cerevisiae: a possible role of N-glycosylation and involvement of the exocyst complex. Genetics *147*, 421–434.

Monzo, P., Gauthier, N.C., Keslair, F., Loubat, A., Field, C.M., Le Marchand-Brustel, Y., and Cormont, M. (2005). Clues to CD2-associated protein involvement in cytokinesis. Mol. Biol. Cell *16*, 2891–2902. 10.1091/mbc.E04-09-0773

Morales, C.P., Holt, S.E., Ouellette, M., Kaur, K.J., Yan, Y., Wilson, K.S., White, M.A., Wright, W.E., and Shay, J.W. (1999). Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. Nat. Genet. *21*, 115–118.

Mullins, J.M., and Biesele, J.J. (1977). Terminal phase of cytokinesis in D-98s cells. J. Cell Biol. *73*, 672–684.

Murthy, M., and Schwarz, T.L. (2004). The exocyst component Sec5 is required for membrane traffic and polarity in the Drosophila ovary. Development *131*, 377–388.

Papoulas, O., Hays, T.S., and Sisson, J.C. (2004). The golgin Lava lamp mediates dynein-based Golgi movements during Drosophila cellularization. Nat. Cell Biol. *7*, 612–618. 10.1038/ncb1264

Paweletz, N. (1967). On the function of the 'Flemming body' during division of animal cells. Naturwissenschaften *54*, 533–535.

Piel, M., Nordberg, J., Euteneuer, U., and Bornens, M. (2001). Centrosome-dependent exit of cytokinesis in animal cells. Science *291*, 1550–1553.

Prigent, M., Dubois, T., Raposo, G., Derrien, V., Tenza, D., Rosse, C., Camonis, J., and Chavrier, P. (2003). ARF6 controls post-endocytic recycling through its downstream exocyst complex effector. J. Cell Biol. *163*, 1111–1121.

Salminen, A., and Novick, P.J. (1989). The Sec15 protein responds to the function of the GTP binding protein, Sec4, to control vesicular traffic in yeast. J. Cell Biol. *109*, 1023–1036.

Saxton, W.M., and McIntosh, J.R. (1987). Interzone microtubule behavior in late anaphase and telophase spindles. J. Cell Biol. *105*, 875–886.

Skop, A.R., Bergmann, D., Mohler, W.A., and White, J.G. (2001). Completion of cytokinesis in C. elegans requires a brefeldin A-sensitive membrane accumulation at the cleavage furrow apex. Curr. Biol. *11*, 735–746.

Skop, A.R., Liu, H., Yates, J., Meyer, B.J., and Heald, R. (2004). Dissection of the mammalian midbody proteome reveals conserved cytokinesis mechanisms. Science *305*, 61–66. Published online May 27, 2004..

Strickland, L.I., and Burgess, D.R. (2004). Pathways for membrane trafficking during cytokinesis. Trends Cell Biol. *14*, 115–118.

Takahashi, N., Hatakeyama, H., Okado, H., Miwa, A., Kishimoto, T., Kojima, T., Abe, T., and Kasai, H. (2004). Sequential exocytosis of insulin granules is associated with redistribution of SNAP25. J. Cell Biol. *165*, 255–262.

Takeda, T., Kawate, T., and Chang, F. (2004). Organization of a sterol-rich membrane domain by cdc15p during cytokinesis in fission yeast. Nat. Cell Biol. *6*, 1142–1144.

Thakur, P., Stevens, D.R., Sheng, Z.H., and Rettig, J. (2004). Effects of PKA-mediated phosphorylation of Snapin on synaptic transmission in cultured hippocampal neurons. J. Neurosci. *24*, 6476–6481.

Thompson, H.M., Skop, A.R., Euteneuer, U., Meyer, B.J., and McNiven, M.A. (2002). The large GTPase dynamin associates with the spindle midzone and is required for cytokinesis. Curr. Biol. *12*, 2111–2117.

Verplank, L., and Li, R. (2005). Cell cycle-regulated trafficking of Chs2 controls actomyosin ring stability during cytokinesis. Mol. Biol. Cell *16*, 2529–2543.

Vites, O., Rhee, J.S., Schwarz, M., Rosenmund, C., and Jahn, R. (2004). Reinvestigation of the role of snapin in neurotransmitter release. J. Biol. Chem. *279*, 26251–26256.

Wang, H., Tang, X., Liu, J., Trautmann, S., Balasundaram, D., McCollum, D., and Balasubramanian, M.K. (2002). The multiprotein exocyst complex is essential for cell separation in Schizosaccharomyces pombe. Mol. Biol. Cell *13*, 515–529.

Wilson, G.M., Fielding, A.B., Simon, G.C., Yu, X., Andrews, P.D., Hames, R.S., Frey, A.M., Peden, A.A., Gould, G.W., and Prekeris, R. (2005). The FIP3-Rab11 protein complex regulates recycling endosome targeting to the cleavage furrow during late cytokinesis. Mol. Biol. Cell *16*, 849–860.

Yeaman, C. (2003). Ultracentrifugation-based approaches to study regulation of Sec6/8 (exocyst) complex function during development of epithelial cell polarity. Methods *30*, 198–206.

Yeaman, C., Grindstaff, K.K., and Nelson, W.J. (2004). Mechanism of recruiting Sec6/8 (exocyst) complex to the apical junctional complex during polarization of epithelial cells. J. Cell Sci. *117*, 559–570.

General information
- List of employees
- News
- Awards & memberships
- Project catalogue
- Core facilities
- Doctoral theses
- Research Foundation
- Vacant positions
- Seminars
- Info for employees

New research building

Webcam photo from construction site (continually updated)

(click to enlarge)

Planned opening: May 1st 2009

Countdown clock:
799d, 18h, 7min, 3sec

Summary of the Report of the Commission of Inquiry

1 Appointment
On 18 January 2006, the Rikshospitalet–Radiumhospitalet Medical Center and the University of Oslo (UiO) jointly appointed a special commission to conduct an independent investigation in accordance with detailed terms of reference.

The background for the investigation was that a researcher employed by these institutions, Jon Sudbø, had admitted fabricating the raw data used for a scientific article published in the renowned medical journal The Lancet in October 2005.

2 The investigation
Early in the investigation it became clear that the entire body of Sudbø's scientific work from 1993-2006 (at least 38 publications) would have to be scrutinized, and that the co-authors (60 altogether) would in reality also have to be subject to investigation. All the authors received a letter requesting them to submit a voluntary written statement, which they all did. Moreover, information was gathered from relevant institutions and other relevant partners. Special mention should be made of the findings from the thorough investigations made by the Cancer Registry of Norway. The Commission also met with individuals and representatives of institutions, including Jon Sudbø. Furthermore, the Commission has obtained documents and other information from several other sources. Available data lists, etc., and published research results have been correlated and compared. Accordingly, the Commission was generally able to judge whether, and the extent to which, the underlying data on which the publications are based are genuine. As its main principle, the Commission has found it appropriate to apply a standard of evidence entailing a qualified preponderance of probability as a condition for accepting a particular fact as grounds for the report.

3 Findings
Jon Sudbø began his PhD project in 1993 under the supervision of Albrecht Reith.

The PhD project consists of two separate parts. One part involves theoretical and applied works on tissue architecture in cancerous tumors and normal tissue. The Commission has not found indications of research flaws related to these works.

As reflected in his subsequent research, most of his PhD project involved characterizing the early stages of oral cancer. The research question was whether and, if so, to what extent, different types of classifications of white patches in the oral cavity were indicative of a high risk for developing oral cancer. The doctoral dissertation and related publications give an affirmative response to this question, asserting that a classification based on DNA content can with great accuracy predict the subsequent development of cancer.

First published in the highly respected New England Journal of Medicine in 2001, this sensational finding was based on DNA analyses of 150 patients with leukoplakia (i.e. 'white patches' that may be early stages of oral cancer) in the oral cavity. In 2004, a second article was published in the New England Journal of Medicine, based on further investigations of the same 150 patients. Based on their own investigations and those made by the Cancer Registry of Norway, the Commission's point of departure is that there are serious problems associated with this crucial patient material. For instance, the same patient appears several times. As far as the Commission can determine, the material consists of 141 different patients at the most, since several patients are represented by several tissue samples that collectively add up to 150. Further, the Commission has found that 69 of the 141 patients included in the study should have been excluded because they had been diagnosed with oral cancer before or at the same time as the leukoplakia was diagnosed. For these patients, it was not possible to study the future development of cancer, since they already had cancer. This error alone is so serious that the results and the conclusions are invalid. The Commission has also uncovered several other inconsistencies. For example, the age distribution in the data files is not consistent with the underlying patient material. Further, the Commission has noted that the reported 150 DNA analyses are to some extent repetitions of data from a far smaller number of patients. The reporting on how DNA analyses and the classification of leukoplakia were

conducted (by several observers) is also incorrect and misleading.

Consequently, the Commission has determined that the data underlying parts of the PhD project, as well as several other publications, are not sufficiently consistent with the actual facts the Commission has found it reasonable to take into account. The internal affairs investigation conducted by the Cancer Registry of Norway has arrived at the same conclusion.

The Commission is of the opinion that the errors and defects that have been exposed are too numerous, too great and too obvious to be attributed to random errors, incompetence or the like; and that the raw data therefore appear to have been fabricated, manipulated and adapted to the desired findings.

The consequence of this is that the doctoral dissertation and three related original articles must be retracted. In addition, subsequent publications must be retracted where they are based on the same raw material, as most of them are. On the same grounds, the Commission also questions one other original article. Further, the Commission has questioned an original article published in the Journal of Clinical Oncology 2005, inter alia in the light of circumstances partially acknowledged by Sudbø. The most recent original article published in The Lancet in 2005 has been retracted, since it is, in its entirety, based on fabricated raw data. Jon Sudbø has admitted this.

This means that the bulk of Jon Sudbø's scientific publications are invalid due to the fabrication and manipulation of the underlying data material.

4 Criticism, possible explanations and preventive measures
The exposed fabrication and manipulation of research data justify criticism against Jon Sudbø. The comments that Sudbø has made to the Commission in a meeting and after having read two draft reports with attached documentation, have not given the Commission reason to make any major changes in the preliminary conclusions drawn during the investigation.

In compliance with the terms of reference, the Commission has posed the question of how such – in retrospect – obvious and gross acts could have been perpetuated over such a long period of time in collaboration with so many well-qualified co-authors/scientists and research institutions.

The Commission points out that there will invariably be certain possibilities for a dishonest researcher to dupe and deceive others. Another factor is that Jon Sudbø has operated relatively independently both as a doctoral candidate and later as a researcher. He has always maintained full and sole control of the underlying data. In that connection, the Commission has found reason to criticize his supervisor for a lack of due diligence and academic supervision during Sudbø's fellowship. This case has also revealed what appears to be a systemic failure at the Norwegian Radium Hospital with respect to a lack of supervision, training and control procedures. Another circumstance is that there has been no formal permission or approval whatsoever of the project on the part of external bodies, nor has anyone taken it upon themselves to arrange for or check this. In this context, it has been noted that the institutions that contributed patient material have not required verification of the necessary permits, e.g. dispensation from mandatory confidentiality.

The Commission has not found indications that others, including some of the co-authors, have been involved in the fabrication and manipulation of research data or by other means been party to scientific misconduct. However, in good conscience and based on cost/benefit considerations, the Commission has not perceived its task as being to investigate less serious types of deviations from the norm. The co-authors can generally be divided into two groups: 1) suppliers (subcontractors), and 2) higher level guarantors (senior researchers), who to little or no degree contributed to or had knowledge of the underlying data material. Most communication has taken place through Jon Sudbø. Thus the co-authors have had little opportunity, as well as little reason, to check the underlying data and each other's contributions. Such a division of labour is not uncommon for medical publications that must necessarily be based on cooperation between researchers with rather dissimilar professional backgrounds and tasks, and thus require that they trust each other.

On the other hand, the Commission has pointed out certain factors to which several people should have reacted, be they co-authors, supervisors, superiors, opponents, colleagues or others. Since there have been a number of less serious mistakes on the part of several people that must be viewed in context (collective and cumulative mistakes), the Commission has found reason to view this as systemic failure, where the responsibility rests with the institutions.

In light of this, the Commission has recommended that the institutions take more responsibility for raising awareness and instructing their researchers about the rules that apply, and that they engage in at least a minimum of verification and control, taking appropriate account of academic freedom.

The Commission has not perceived its task as being to expose specific damaging effects. This will probably be a topic for a subsequent investigation by the Norwegian Board of Health. Notwithstanding, the Commission has noted that colleagues, researchers, clinicians and individual patients have probably used Sudbø's research results, and it is therefore reasonable to assume that some of them have been affected. The serious implications of this must have been obvious to Jon Sudbø right from the start.

5 The Commission's Report – an overview
Chapter 2 of the investigative report presents the conditions of the Commission's appointment, the terms of reference and methods of working. The chapter discusses the investigative principle adopted, mode of information retrieval, the principle of contradiction, standards of evidence, the relationship to disclosure, and thresholds for criticism.

In Chapter 3, the Commission has found reason to outline the ethical and legal framework that applies to medical and health research. Here, the Commission provides a general review of the rules of authorship and supervision, etc.

Chapter 4 reviews the facts the Commission has chosen to take into account. The facts are presented in chronological order, beginning with Jon Sudbø's PhD project, which commenced in 1993. There is an explanation of the raw data underlying parts of Jon Sudbø's doctorate and several subsequent publications. The Commission discusses in detail which patient data Sudbø actually had or may have had, comparing it with the data Sudbø and his co-authors stated that they have had in different publications. The Commission then reviewed Sudbø's subsequent scientific publications, which are mainly based on the original raw data from the PhD project.

In Chapter 5, the Commission has attempted to illuminate certain circumstances that may help explain how and why things turned out the way they did.

Chapter 6 offers a brief discussion of the possible consequences of the situation, not least for Norwegian research and patients.

Chapter 7 summarizes the findings and the circumstances worthy of criticism which the Commission has found reason to point out. This criticism refers to individuals and institutions alike.

Finally, the Commission has made certain recommendations in Chapter 8 by way of conclusion.

Link to PDF document of the complete report

Editorial staff | Sitemap | Terms of use
The Norwegian Radium Hospital, 0310 Oslo, Norway
Webmaster: Trond Olav Berg, email: t.o.berg@labmed.uio.no
Phone switchboard: +47 22 93 40 00